

AD _____

Award Number: DAMD17-99-1-9457

TITLE: Tumor Specific Gene Expression and Tumor Specific Vector
Replication for Systemic Chemotherapy Sensitization
Treatment of Breast Cancer

PRINCIPAL INVESTIGATOR: Albert B. Deisseroth, M.D., Ph.D.

CONTRACTING ORGANIZATION: Sidney Kimmel Cancer Center
San Diego, California 92121

REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030328 296

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 01 - 30 Jun 02)	
4. TITLE AND SUBTITLE Tumor Specific Gene Expression and Tumor Specific Vector Replication for Systemic Chemotherapy Sensitization Treatment of Breast Cancer			5. FUNDING NUMBERS DAMD17-99-1-9457	
6. AUTHOR(S) : Albert B. Deisseroth, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Sidney Kimmel Cancer Center San Diego, California 92121 E*Mail: adeisseroth@skcc.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
14. ABSTRACT Once breast cancer recurs after initial surgery or radiation therapy and is found to be incompletely responsive to salvage chemotherapy, there is no established treatment that can prolong survival. To address this problem, the Deisseroth laboratory has developed a series of vectors which are directed to the use of the L-plastin tumor specific transcriptional promoter to control the expression of a chemotherapy sensitization gene (cytosine deaminase) and a viral replication gene (E1A) so that any toxic effect is tumor specific. These vectors have been shown to suppress the growth of human breast cancer cell lines in a SCID mouse model, and to produce a direct cytolytic effect on breast cancer cell lines which is not seen in explant cultures of normal breast epithelial cells. By combining the E1A and the CD transcription units in a single vector we have shown that this vector is superior to vectors with a single E1A or CD transcription unit. We will now complete the steps necessary for this vector to be available for therapy and will study the use of this vector in combination with chemotherapy as a means to directly kill breast cancer cells and to control metastatic breast cancer.				
14. SUBJECT TERMS adenoviral vector, breast cancer, cytosine deaminase, 5FU			15. NUMBER OF PAGES 66	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	8
Appendices.....	9

Introduction:

This report summarizes progress on a project funded by the US Army Breast Cancer Research Fund (DAMD 17-00-9457), which is designed to develop a new approach to the treatment of breast cancer. We have placed two transcription units under the control of the L-plastin tumor specific promoter: E1A and the cytosine deaminase (CD) chemotherapy sensitization gene. We have shown that this vector is superior to those with only one transcription unit. We have shown that this is the case in two types of settings: Breast cancer and colon cancer. During this past year, two manuscripts have been accepted for publication on this work. The data presented in this progress report shows that the two gene transcription unit vector (Lp-CD-E1A) is superior to the either of the single transcription unit vectors.

Body: Narrative Description of the Results During the Reporting Period of Funding: July 1, 2001-June 30, 2002

Injae Chung from our laboratory was the first to show that a truncated L-plastin promoter could produce high expression of transgenes in a tumor specific manner when placed in the adenoviral vector backbone. Chung showed (Cancer Gene Therapy 6: 99-106, 1999) that infection of explants of ovarian cancer cells with the AdVLpLacZ resulted in high expression levels of beta-galactosidase, whereas no LacZ gene expression occurred in explants of normal peritoneal epithelial cells (see Figure 1 below).

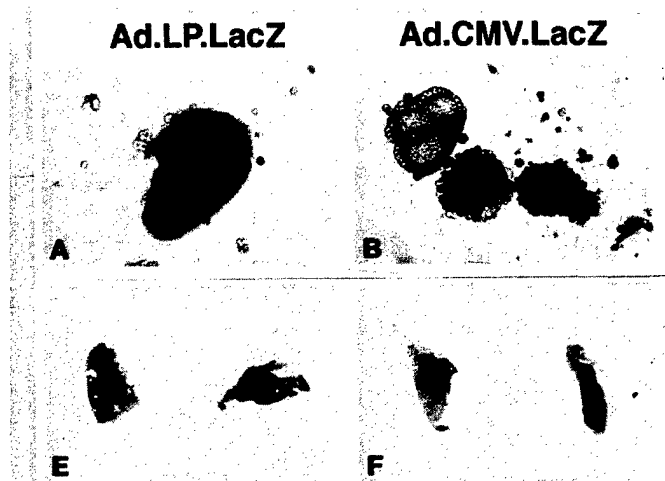


Figure 1 Panel A and B: Ascitic ovarian cancer cells infected with either the AD.LP.LacZ or the AD.CMV.LacZ vector. Panel E and F: Biopsy cells from the mesothelium of patients undergoing surgical procedures exposed to either the AD.LP.LacZ or the AD.CMV.LacZ vector.

XY Peng in our laboratory then showed (Cancer Research 61: 4405-4413, 2001)) that AdV with the LacZ reporter gene under the control of the L-plastin promoter (AdLpLacZ) produced beta-galactosidase activity in breast and ovarian cancer cell lines and explants of ovarian cancer (see Table I below), but not in infected explants of normal peritoneal cells nor in organ cultures of normal ovarian epithelial cells. She showed that there was much more toxicity of the AdLpCD vector when used in vitro with 5FC to explants of ovarian cancer than there was to explants of normal peritoneal tissue (see Table II below). In addition, the use of a replication incompetent AdV carrying a cytosine deaminase transcription unit under the

control of the L-plastin promoter prevented engraftment of the Ovar-5 and Skvo3 human ovarian cancer cell lines in the peritoneal cavity of nude mice (see Table III below).

Table I Cells + for Beta-Galactosidase

Vector	Ovar. Ca.	Periton. Cells
AdCMVLacZ	50-90%	60-80%
AdLpLacZ	15-50%	1-4%

Table II Cell Death After Vector/5FC
% Cells Killed

Cells	AdCMVCD	ALpCD
Ovarian Ca.	90%	75%
Periton. Cells	95%	10%

Table III: % Animals + for Tumor

Cell Lines	AdLpLacZ	AdLpCD
Ovcar5	10/10	0/10
Skov3	5/5	0/5

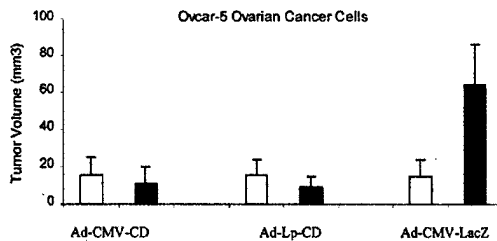


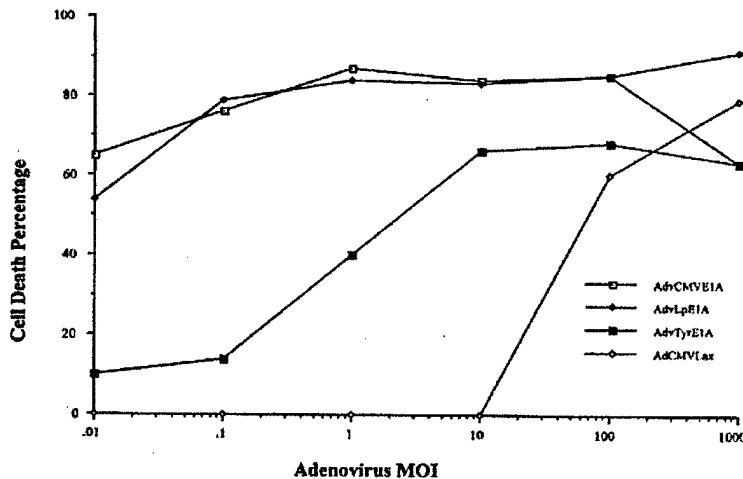
Fig. 2 Effect of *in vivo* injection of tumor nodules with adenoviral vectors.

There was no toxicity to the normal cells of the peritoneum. Finally, intratumoral injection of the AdLpCD vector suppressed the growth of ovarian cancer tumor nodules (see Figure 2).

Lixin Zhang of our laboratory created a series of AdV carrying the E1A gene under the control of the L-plastin promoter (AdVLpE1A). His

experiments, which have been summarized in manuscript which has been published by the Journal of Molecular Therapy (Mol. Therapy. 6: 386-393, 2002), showed that the AdVLpE1A vector was 1000 times more toxic to explant cultures of ovarian carcinoma than a vector carrying a LacZ transcription unit (see Figure 3 below and Figure 4 below).

Figure 3



This AdLpE1A was as toxic to explants of ovarian cancer (see Figure 3) and as was the AdCMVE1A vector (see Figure 3). The AdLpE1A was not toxic to explants of normal breast epithelial cells (see Figure 5 below) whereas both the AdCMVE1A vector and the wild type adenovirus were toxic. This showed that Zhang's cytolytic vectors with the L-plastin promoter were tumor specific. Finally Zhang showed that the AdLpE1A suppressed human tumor xenografts of human breast

cancer cell lines (both MCF-7 and MBAMD468) in SCID mice (see Figure 6 below).

Figure 4

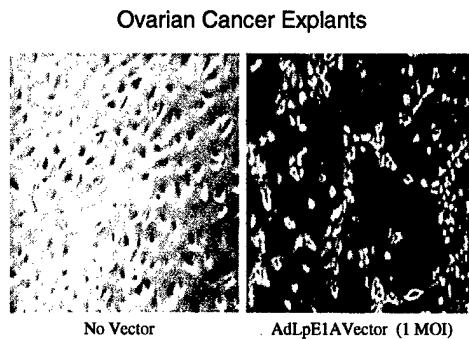


Figure 5

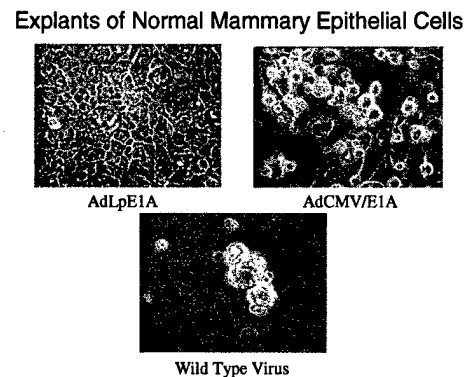
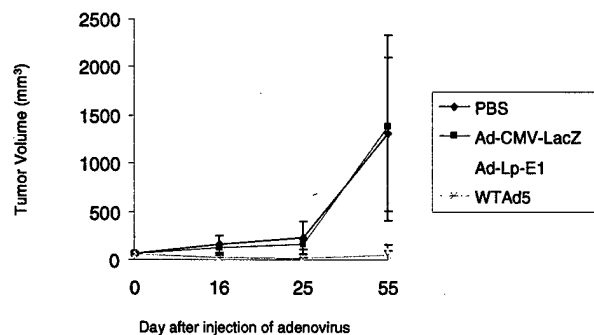


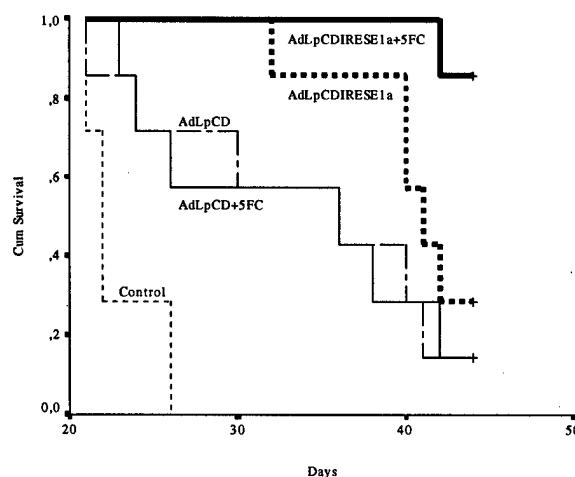
Figure 6



AdVLpCDIRESE1A was the most effective vector against the colonic cancer cell lines and much more effective than the single transcription unit vectors.

The next step in the development of the AdV in the Deisseroth laboratory was the construction by Lixin Zhang of an AdV in which the L-plastin promoter regulated a bicistronic transcription unit composed of the cytosine deaminase gene linked to the E1A gene by an IRES element. These vectors were tested by Hakan Akbulut, of our laboratory, in collaboration with Lixin Zhang. The AdVLpCDIRESE1A vector was first compared in vitro with the AdVLpE1A vector and the AdVLpCD vector. The

Figure 7



Then, Akbulut studied intratumoral injection of the AdVLpCDIRESE1A with and without 5FC as compared to the AdLpCD with and without 5FC (Akbulut et al: The efficiency of replication-competent adenoviral vectors carrying L-plastin promoted cytosine deaminase gene in colon cancer)). Again, the intratumoral injection of the bicistronic vector with 5FC was much more effective in suppressing the growth of the

human tumor xenograft than was the single transcription unit vector (see Figure 7). The work proposed in the research grant application is designed to complete the final step in the development of the adenoviral vectors for use as intracavitary therapy for ovarian cancer by modifying the fibrillar protein to target the adenoviral infection to ovarian cancer cells.

Key Research Accomplishments:

- A. Development of a bi-cistronic transcription unit vector;
- B. Demonstration that this vector is more effective in suppressing cancer than either of the single transcription units

Reportable Outcomes:

Papers Completed During the Funding Period

- a. Peng XY, Rutherford T, Won JH, Pizzorno G, Zeltermann D, Sapi E, Kaczinski B, Leavitt J, Crystal R, and Deisseroth A. L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer. *Cancer Research* 61: 4405-4413, 2001.
- b. Deisseroth A, Fujii T, Peng XY, Austin D, Rutherford T, Brandsma J, and Schwartz PE. Molecular Chemotherapy in CME *Journal of Gynecologic Oncology* 6:23-28, 2001.
- c. Zhang L, Akbulut H, Tang YC, Peng XY, Pizzorno G, Sapi E, Manegold S and Deisseroth A. Adneoviral vectors with E1A regulated by tumor specific promoters are selectively cytolytic for breast cancer and melanoma. *Molecular Therapy* 6: 386-393, 2002.
- d. Akbulut H, Zhang L, and Deisseroth A. The efficiency of replication-competent adenoviral vectors carrying the L-plastin promoted cytosine deaminase gene in colon cancer. *Cancer Gene Therapy*, In Press, 2002.
- e. Akbulut H and Deisseroth A. Prevention of Cervical Cancer: Chemoprevention. In *Progress in Oncology* 2002. Eds: DeVita, Hellman and Rosenberg. Jones and Bartlett Publishers, Sudbury, MA, 251-269, 2002.

Conclusions:

The initial strategy designed to create a conditionally replication competent which was directly cytolytic on the basis of replication and was also sensitizing to chemotherapy, was shown to be successful. We are now in the phase of completing the preclinical in vivo experiments necessary to prepare for a clinical trial.

References: None

Appendices: Manuscripts

Peng XY, Rutherford T, Won JH, Pizzorno G, Zeltermann D, Sapi E, Kaczinski B, Leavitt J, Crystal R, and Deisseroth A. L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer. *Cancer Research* 61: 4405-4413, 2001.

Deisseroth A, Fujii T, Peng XY, Austin D, Rutherford T, Brandsma J, and Schwartz PE. Molecular Chemotherapy in CME *Journal of Gynecologic Oncology* 6:23-28, 2001.

Zhang L, Akbulut H, Tang YC, Peng XY, Pizzorno G, Sapi E, Manegold S and Deisseroth A. Adenoviral vectors with E1A regulated by tumor specific promoters are selectively cytolytic for breast cancer and melanoma. *Molecular Therapy* 6: 386-393, 2002.

Akbulut H, Zhang L, and Deisseroth A. The efficiency of replication-competent adenoviral vectors carrying the L-plastin promoted cytosine deaminase gene in colon cancer. *Cancer Gene Therapy*, In Press, 2002.

Akbulut H and Deisseroth A. Prevention of Cervical Cancer: Chemoprevention. In *Progress in Oncology* 2002. Eds: DeVita, Hellman and Rosenberg. Jones and Bartlett Publishers, Sudbury, MA, 251-269, 2002.

The Use of the L-Plastin Promoter for Adenoviral-mediated, Tumor-specific Gene Expression in Ovarian and Bladder Cancer Cell Lines¹

Xue Yan Peng, Jong Ho Won, Thomas Rutherford, Takuma Fujii, Daniel Zeltermann, Giuseppe Pizzorno, Eva Sapi, John Leavitt, Barry Kacinski, Ronald Crystal, Peter Schwartz, and Albert Deisseroth²

Genetic Therapy Program, Yale Cancer Center [X. Y. P., J. H. W., T. F., D. Z., G. P., J. L., A. D.], Medical Oncology Section, Departments of Internal Medicine, Therapeutic Radiology Program [E. S., B. K.], and Obstetrics and Gynecology [T. R., P. S.], Yale University School of Medicine, New Haven, Connecticut 06520, and Cornell Medical School, New York, New York, 10021 [R. C.]

ABSTRACT

A 2.4-kb truncated L-plastin promoter was inserted either 5' to the *LacZ* gene (Ad-Lp-LacZ) or 5' to the cytosine deaminase (*CD*) gene (Ad-Lp-CD) in a replication-incompetent adenoviral vector backbone. Infectivity and cytotoxicity experiments with the LacZ and CD vectors suggested that the L-plastin promoter-driven transcriptional units were expressed at much higher levels in explants of ovarian cancer cells from patients and in established ovarian or bladder cancer cell lines than they were in normal peritoneal mesothelial cells from surgical specimens, in organ cultures of normal ovarian cells, or in the established CCD minimal deviation fibroblast cell line. Control experiments showed that this difference was not attributable to the lack of infectivity of the normal peritoneal cells, the normal ovarian cells, or the minimal deviation CCD fibroblast cell line, because these cells showed expression of the *LacZ* reporter gene when exposed to the replication-incompetent adenoviral vector carrying the cytomegalovirus (CMV)-driven *LacZ* gene (Ad-CMV-LacZ). The Ovar-5 and Skov-3 ovarian cancer cell lines exposed to the Ad-Lp-CD adenoviral vector were much more sensitive to the prodrug 5-fluorocytosine (5FC), which is converted from the 5FC prodrug into the toxic chemical 5-fluorouracil, than was the CCD minimal deviation fibroblast cell line after exposure to the same vector. A mouse xenograft model was used to show that the Ad-Lp-CD vector/5FC system could prevent engraftment of ovarian cancer cells in nude mice. Finally, injection of the Ad-Lp-CD vector into s.c. tumor nodules generated a greater reduction of the size of the tumor nodules than did injection of the Ad-CMV-LacZ vectors into tumor nodules. The Ad-Lp-CD vectors were as suppressive to tumor growth as the Ad-CMV-CD vectors. These results suggest that an adenoviral vector carrying the *CD* gene controlled by the L-plastin promoter (Ad-Lp-CD) may be of potential value for the i.p. therapy of ovarian cancer.

INTRODUCTION

Adenoviral vectors are currently among the most frequently used vectors in the gene therapy of cancer because of their high titers, ease of production, high infection efficiency for epithelial neoplastic cells, and the fact that their transcriptional units can be expressed extrachromosomally in nondividing cells. A possible disadvantage of this vector is that its broad host range also results in infection of both the intended tumor cells as well as of the surrounding normal tissues (1-3). This limits the utility of these vectors, especially when the vector gene products are designed to sensitize tumor cells to chemotherapy or to radiation therapy, because of the unwanted toxicity thereby generated in the normal cells.

One way to circumvent this limitation would be to use a tissue-specific transcriptional promoter active only in the target tumor cells. Our laboratory has constructed adenoviral vectors in which the L-plastin promoter is used to activate the expression of therapeutic transgenes in neoplastic but not in normal epithelial cells. L-plastin, which belongs to a family of genes which encode actin-binding proteins, was discovered by Leavitt (4) and his colleagues (5, 6). The only normal cell in which this protein is detectable is the mature leukocyte. This protein has been demonstrated to be present in >90% of epithelial neoplastic cells and is not found in normal epithelial cells. Therefore, the L-plastin promoter may be of potential utility in cancer gene therapy because it can be used to drive the expression of heterologous genes in a tumor-specific manner in the context of recombinant adenoviral vectors. Chung *et al.*, in our laboratory, had reported previously that the *LacZ* gene, when driven by the L-plastin promoter, is expressed in ovarian cancer cells, but not in normal mesothelial peritoneal cells, obtained at the time of surgical resection of ovarian cancer from patients (7).

We now are reporting the results of experiments based on replication-deficient adenoviral vectors that contain either a *LacZ* reporter gene or a *CD*³ therapeutic transcriptional unit regulated by a 2.4-kb fragment of the L-plastin promoter in bladder and ovarian cancer cell lines, in explants of normal and neoplastic ovarian primary tissue in organ culture, and in ovarian cancer established cell lines in a nude mouse-human tumor xenograft animal model. *CD* is a bacterial gene which converts 5FC, which is nontoxic to cell lines and primary cells, into 5FU, a compound which is toxic to most cells (2, 8). The levels of phosphorylated 5FU generated within *CD*-positive cells are sufficiently high that even nondividing cells die because of disruption of mRNA processing and protein synthesis.

The results of these experiments have shown that:

(a) the level of the L-plastin promoter driven the *LacZ* heterologous reporter gene expression is lower in an established minimal deviation fibroblast cell line (CCD) when compared with a collection of established epithelial tumor cell lines derived from ovarian cancer and bladder cancer;

(b) the L-plastin promoter activates the *LacZ* and *CD* transcriptional units to a higher level in ovarian cancer cells than in monolayer and organ explant cultures of normal ovarian tissue or of normal peritoneal tissue; and

(c) the cytotoxic effect of replication-incompetent adenoviral vectors carrying the *CD* transcriptional unit driven by the L-plastin promoter is greater to ovarian cancer cells exposed *in vitro* to 5FC than to explants of normal peritoneum. In addition, the suppressive effect of the L-plastin-driven *CD* vectors on the *in vivo* growth of ovarian cancer cell lines is equal to that of the CMV-driven *CD*

Received 2/3/00; accepted 4/10/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹Supported by BC980260 from the United States Army Breast Cancer Research Program, the Breast Cancer Women's League of Greenwich Connecticut, the Hull Development Fund of the Yale Cancer Center, the Bush Leukemia Research Fund, the American Cancer Society, The Audrey Demas Melanoma Research Fund, the Breast Cancer Research Foundation, and the Ensign Professorship in Medicine at Yale (to A. D.).

²To whom requests for reprints should be addressed, at Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8032. Phone: (203) 737-5608; Fax: (203) 737-5698; E-mail: albert.deisseroth@yale.edu.

³The abbreviations used are: *CD*, cytosine deaminase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; CMV, cytomegalovirus; NBSC, new born calf serum; CAR, coxsackie B/adenovirus receptor; pfu, plaque-forming units; MOI, multiplicity of infection; X-Gal, (5-bromo-4-chloro-3-indolyl)- β -D-galactopyranoside; ONPG, *O*-nitrophenyl- β -D-galactopyranoside.

vectors. These results suggest that adenoviral vectors carrying the CD transcription unit driven by the L-plastin promoter may be of use in the i.p. treatment of metastatic ovarian cancer.

MATERIALS AND METHODS

Cells and Cell Culture

Human bladder carcinoma cell lines (J82 and EJ) were obtained from Dr. Richard Cote of the University of Southern California, Los Angeles, CA. The CCD minimal deviation human fibroblast cell line, the 293 transformed human kidney cell line, and the Skov-3 human ovarian cancer cell line were obtained from American Type Culture Collection. The Hey cystadenocarcinoma papillary ovarian cancer cell line was obtained from Eva Sapi of the Department of Therapeutic Radiology at Yale University (New Haven, CT). J82, EJ, Hey, and 293 cells were propagated in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS obtained from Hyclone Laboratories, Inc. (Logan, UT). The Ovar-5 human epithelial ovary carcinoma cell line was obtained from Dr. Thomas C. Hamilton of the Fox Chase Cancer Center, Philadelphia, PA. Ovar-5 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS. The Skov-3 human ovarian adenocarcinoma cell line was propagated in McCoy5A medium supplemented with 10% heat-inactivated NBCS. All cell cultures were maintained in a 5% CO₂, humidified tissue culture incubator at 37°C.

Chemicals and Reagents

5-FC, 5FU, fluorescein di- β -D-galactopyranose, and X-Gal were purchased from Sigma Chemical Co. The β -Galactosidase Assay Kit was purchased from Stratagene Company. 6-³(H)5-fluorocytosine (4.1 Ci/mmol) and 6-³(H)5-fluorouracil were purchased from Noravak Biochemicals Inc. of Brea, CA. Monoclonal antibodies to $\alpha\beta$ 3 (LM609) and $\alpha\beta$ 5 (PIF6) integrins were purchased from Chemo-Con International. A monoclonal antibody to the CAR, which binds the adenoviral fibrillar protein, was obtained from Dr. R. W. Finberg of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

Construction of Replication-incompetent Recombinant Adenoviral Vectors

The Ad-CMV-CD vector, which contained the CD gene controlled by a CMV promoter (7) in place of the adenoviral *E1A* and *E1b* genes, was obtained from the laboratory of Dr. Ron Crystal of the Cornell Medical School, New York, NY (8, 9). A similar adenoviral vector (Ad-CMV-LacZ) was engineered in our laboratory in which a β -galactosidase transcriptional unit was inserted into the *E1a* and *E1b* regions of the adenoviral vector backbone (7). Injae Chung of our laboratory truncated the 5-kb L-plastin promoter to a 2.4-kb fragment, which extended from nucleotide -2265 of the 5' region of the L-plastin promoter to +18 bp from the transcription initiation site of the *L-plastin* gene (7). The number of infectious adenoviral particles, expressed as plaque-forming units (pfu) present in the viral stocks, was determined by limiting dilution assay of plaque formation in 293 cells exposed to various dilutions of the vector (10, 11).

Analysis of Cellular Receptors on Tumor Cells That Participate in Vector Uptake

Mouse monoclonal antibodies to the $\alpha\beta$ 3 (LM609) integrin and the $\alpha\beta$ 5 (PIF6) integrin and to the CAR receptors were used to detect the density of the human $\alpha\beta$ 3, $\alpha\beta$ 5, and CAR receptors on the test cells. The FACS Star Flow Cytometer (Becton Dickinson) in the Yale Cancer Center FACS Core Laboratory (New Haven, CT) was used to determine the percentage of cells positive for each receptor.

β -Galactosidase Activity Assay

X-Gal Staining. Cells were washed in PBS, trypsinized, and the viable cell number determined by trypan blue exclusion using a light microscope. Cells (3×10^5) for each cell line were infected with varying ratios of pfu/cell (MOI) of the vector in DMEM supplemented with 2% NBCS for 90 min. After this,

the cells were plated in six-well plates in complete medium in duplicate cultures. After 48 h of incubation at 37°C in a 5% CO₂, humidified tissue culture incubator, the cells were fixed with ice-cold 2% paraformaldehyde/0.2% glutaraldehyde for 10 min. The level of β -Gal-expression cells was then assessed by staining the cultures with X-Gal and potassium-ferricyanide/ferrocyanide solution essentially as described previously (12, 13). The average number of β -Gal-expressing (blue) cells/well was determined by counting five separate microscopic high-power fields.

β -Galactosidase Assay (ONPG). Cells (5×10^5) were infected at 20 MOI with Ad-Lp-LacZ or Ad-CMV-LacZ in 2% serum for 90 min. PBS was used to wash the cells, which were seeded in six-well plates with the fresh culture medium. The cells were then incubated for 48 h, after which the β -galactosidase assay was conducted (β -Galactosidase Assay Kit, Stratagene). Briefly, the cells were washed in PBS and lysed in 200 μ l lysis buffer and the cell debris removed by centrifugation for 5 min. The cell lysate was diluted 10 times, and 15 μ l of the cell lysate were pipetted into a 96-well microtiter dish, 145 μ l of buffer A- β -mercaptoethanol mixture was added to each well with subsequent incubation for 5 min at 37°C. Fifty μ l of ONPG were added to each well, and the dish was incubated at 37°C for 25 min; the mixtures turned bright yellow. The reaction was terminated by adding 90 μ l of stop solution and the microtiter dish was scanned in the microtiter dish reader set at 405 nm, and the absorbance (OD) was determined.

The Effect of 5-FU Released from CD Vector-infected Cells on Uninfected Cells

To quantify the effect of 5-FU released from infected cells on uninfected cells, different cell lines were infected at varying MOI (20 MOI, 80 MOI, and 160 MOI) using the Ad-CMV-CD or Ad-Lp-CD vectors. The infected cells and the noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells (14, 15). Cells were then seeded in duplicate in six-well tissue culture plates and incubated for 24 h with subsequent incubation with 500 μ M/liter 5FC for 5 days. The number of surviving cells was determined using trypan blue exclusion.

Comparison of the 5-FU Sensitivity (IC₅₀) of Ovarian Cancer and Bladder Cancer Cell Lines with CCD (Minimal Deviation Fibroblast Cell Lines)

The concentrations of 5FU used for the cytotoxicity test (IC₅₀) were 100, 50, 10, 1, and 0.5 μ M. After 96 h, the cells were removed with trypsin-EDTA and the cell number calculated using the Coulter Counter ZM (Hialeah, FL).

The Toxicity of Adenoviral Vectors

Cells (2×10^5) were infected with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD, or Ad-Lp-CD vectors at MOI of 0, 5, 20, 40, 80, and 160 for 90 min and then seeded in six-well plates in duplicate. Twenty-four h later, 0.5 mM 5FC was added to each well, and then the cells were incubated for 5 days. Then the cells were trypsinized, and the surviving cells were counted using trypan blue exclusion (16). We arbitrarily assigned a 100% value to the cells incubated at 0 MOI and calculated the percentage of viable cells in the cultures to which vector had been added.

Vector Studies in Monolayer Explant Culture

Biopsy samples were cut into small pieces. These pieces were then digested with collagenase to disaggregate the tissue. To test the sensitivity of the patient samples to infection and 5FC sensitization with the Ad-CMV-CD and Ad-Lp-CD vectors, the cells were grown in T25 flasks to 90% confluence. Then the cells were washed in PBS and exposed to vector directly for 90 min in the flasks containing DMEM supplemented with 2% NBCS. Then the cells were incubated for 5 days at 500 μ M/liter 5FC concentration, and the cell viability was determined by light microscopic examination.

Vector Studies on Organ Culture of Ovarian Cancer and Normal Ovarian Tissue

Each specimen was cut into pieces of approximately 1–2 mm³ and immersed in 4 ml of DMEM:Ham's F12 medium, which was supplemented with 10% charcoal-stripped serum (17). Cultures were incubated at 37°C in six-well

plates on a shaking platform for 24–48 h, after which the tissues were exposed to the Ad-CMV-LacZ or Ad-Lp-LacZ viral vectors for 90 min in serum-free medium. The tissues were washed with PBS. Then tissues were incubated for 48 h in fresh culture medium. The tissues were then frozen in OCT, and X-Gal staining was used to measure the Ad-CMV-LacZ and Ad-Lp-LacZ expression on the section slides.

Studies of *in Vitro* Vector-infected Ovarian Cancer Cell Line in Nude Mice

Ovar-5 tumor cell lines were infected *in vitro* at 100 MOI with either the Ad-Lp-LacZ or Ad-Lp-CD adenoviral vectors for 60 min, washed with PBS, and then resuspended in PBS (4×10^7 cells/1 ml PBS). Ten female nude mice 6–8 weeks of age (25–28 grams in weight), which were purchased from Cox, Inc., Cambridge, MA, were injected i.p. with 40 million Ovar-5 ovarian carcinoma cells previously infected at 100 MOI with the Ad-Lp-LacZ vector. An additional 10 26–28-gram mice 6–8 weeks of age were injected i.p. with Ad-Lp-CD-infected cells. From the second day, all 20 of the mice were injected once a day with 5FC at 500 mg/kg i.p. for 10 days. Three weeks after tumor cell injection, the 10 Ad-Lp-LacZ-injected mice and 7 of the Ad-Lp-CD-injected mice were killed and autopsied. At the 50th day, another three Ad-Lp-CD-injected mice were killed and autopsied.

In other experiments, five female nude mice were injected i.p. with 40 million Skov-3 cells previously infected *in vitro* with the Ad-Lp-CD vector at 80 MOI. Another five mice were injected i.p. with Skov-3 cells previously infected *in vitro* at 80 MOI with the Ad-Lp-LacZ vectors. Then all of the 10 mice were injected i.p. with 500 mg/kg of 5FC daily for 10 days. Three weeks later, the mice were killed and autopsied (18, 19).

Studies of *in Vivo* Intratumoral Injection of Adenoviral Vectors

EJ cells (5×10^6) in PBS were injected s.c. in 25 nude mice. Three weeks later, the tumor size (width and length) was measured, then the tumor volume (mm^3) was calculated according to the formula: Tumor volume = length \times width²/2 (20, 21). Then, tumor nodules in eight mice were injected with 10^8 pfu of the Ad-CMV-CD virus. Tumor nodules in an additional eight mice were injected with 10^8 pfu of the Ad-Lp-CD virus, and tumor nodules in another nine mice were injected with 10^8 pfu of the Ad-CMV-LacZ virus. After this, 500 mg/kg of 5FC was injected into the peritoneal cavity each day, once a day, for 5 days. Two weeks later, we measured the tumor size again and compared the tumor growth before and after the treatment with viral particles and 5FC. Another 20 nude mice were injected s.c. with 5×10^6 Ovar-5 tumor cells. After this, the same vector injections and 5FC treatments were conducted as for the EJ tumor cell in the nude mice. Autopsy of the mice was carried out, and H&E-stained sections of the tumor and the adjacent tissues were examined to measure the toxicity of the vectors.

RESULTS

Study of Factors Affecting Percentage of β -Galactosidase-positive Cells after Exposure to the Ad-CMV-LacZ or Ad-Lp-LacZ Vectors. The infectivity of cell lines by adenoviral vectors has been reported to be dependent on the presence of the CAR, which mediates the binding of the vector to the target cell (22–24), the level and functional state of both the $\alpha\beta3$ and $\alpha\beta5$ integrin receptors, which are important for endocytosis of the vector, and the release of the vector from the endosome (25–27). Cell lines in which the $\alpha\beta3$ receptors are low or functionally inactive may have low levels of expression of vector transgenes, because the amount of vector DNA reaching the nucleus, where it is transcribed into mRNA, will be reduced in $\alpha\beta$ -deficient cell lines because of sequestration in the endosome.

To study the effect of these receptors on the uptake of the adenoviral vector into cancer cell lines and the subsequent expression of its LacZ transgene in target cells, the Ovar-5, Hey, and Skov-3 ovarian cancer cell lines, the EJ, and J82 bladder cancer cell lines, and the CDD minimal deviation cancer cell line were exposed to the Ad-CMV-LacZ vector. Then these cell lines were studied for the percent-

age of cells that were positive for β -galactosidase. We chose a vector with the CMV promoter, because this promoter is known to be active in most, if not all, mammalian cells. Differences in β -galactosidase in these cell lines would therefore be attributable to differences in binding and endocytosis of the vector or release of the vector from the post-entry endosome. As shown in Table 1, the cells of all of the established ovarian and bladder cancer cell lines studied had a high percentage of cells positive for the CAR receptor (except for the Hey ovarian carcinoma cell line, in which none of the cells were detectable as positive for CAR). Among the established carcinoma cell lines in which a high percentage of cells were positive for CAR, all of the cell lines except for the Ovar-5 cell line had >80% of the cells positive for the $\alpha\beta5$ receptor. The percentage of Ovar-5 cells positive for the $\alpha\beta5$ integrin receptor was 57%. The percentage of cells positive for the $\alpha\beta3$ integrin receptor was more variable among the cell lines. Only one-half of the Ovar-5 cells were positive for the either of the integrin receptors.

Not surprisingly, a high percentage of the cells of all of the established tumor cell lines studied, except for the Hey cell line, were detectable as positive for β -galactosidase after exposure to the Ad-CMV-LacZ vector (see Table 1). This suggests that cell lines in which a high percentage of cells are positive for both the CAR and the $\alpha\beta5$ integrin receptors will be infectible by the adenoviral vectors and therefore will score positive for the protein product of a vector transgene if the transcriptional promoter driving the expression of the transgene is very strong, as is the case with the CMV promoter. Surprisingly, as shown in Table 1, although only 30% of the cells of the CCD cell line are positive for the CAR receptor and only 63% of the CCD cells were positive for the $\alpha\beta3$ integrin receptors, up to 70% of the CCD cells are positive for β -galactosidase after exposure to the Ad-CMV-LacZ vector. Experiments carried out previously in our laboratory have shown that the CCD cell line is infectible by the Ad-CMV-LacZ vector (7). This suggests that there may be a CAR-independent mechanism of binding of the adenoviral vector to the CCD cells, and that the strength of a transcriptional promoter may overcome in part the limitation imposed on transgene expression by a lower level of the $\alpha\beta3$ receptor.

Comparison of LacZ Gene Expression Levels in Cell Lines Infected with Either the Ad-CMV-LacZ or the Ad-Lp-LacZ Vectors. Another factor that may alter the percentage of cells scoring positive for transgene expression after exposure to an adenoviral vector is the level of activity of the transcriptional promoter regulating the vector transgenes in these different cell lines. Because it had been reported that the *L-plastin* gene was detectable in most tumor cell lines, but not in any normal cells of the body except for the mature leukocyte (4, 6), the same cell lines exposed to the Ad-CMV-LacZ vector were also exposed to an adenoviral vector in which the LacZ gene was regulated by the *L-plastin* promoter (Ad-Lp-LacZ).

Table 1 Characterization of percentage of cells positive for the CAR, $\alpha\beta3$, and $\alpha\beta5$ receptors as measured by FACS analysis and study of infectivity of cells by Ad-CMV-LacZ Vector at 20 MOI as measured by β -galactosidase assay (X-Gal)

$\alpha\beta3$, $\alpha\beta5$, and CAR receptor levels were measured by mouse monoclonal antibodies, and the FITC-conjugated antimouse antibody was used to stain the cells. Then, FACS analysis was used to detect the percentage of the receptor-positive cells ($n = 2$). For infectivity, cells were exposed to virus in serum-free medium for 90 min at 20 MOI and incubated for 48 h in culture medium. Then, cells were stained by X-Gal analysis ($n = 2$).

	$\alpha\beta3$	$\alpha\beta5$	CAR	β -Gal
EJ	83 \pm 8	82 \pm 5	95 \pm 8	95 \pm 8
J82	56 \pm 6	78 \pm 7	80 \pm 10	88 \pm 10
Skov-3	64 \pm 6	91 \pm 8	87 \pm 7	85 \pm 11
Ovar-5	48 \pm 7	57 \pm 5	88 \pm 10	65 \pm 8
Hey	81 \pm 5	96 \pm 10	0	10 \pm 4
CCD	63 \pm 8	93 \pm 4	29 \pm 5	70 \pm 9

Table 2 Comparison of β -galactosidase levels in cell line exposed to Ad-CMV-LacZ or Ad-Lp-LacZ (ONPG, OD)

Cells were exposed in serum-free conditions for 90 min at 20 MOI. After 48 h of incubation in culture medium, the level of the β -galactosidase (ONPG) in each cell line was measured by optical density, as outlined in "Materials and Methods" ($n = 2$).

	Ad-CMV-LacZ	Ad-Lp-LacZ	Ratio of CMV/Lp
EJ	1.1 \pm 0.2	0.9 \pm 0.1	1.2
J82	1.0 \pm 0.1	0.4 \pm 0.1	2.5
Skov-3	0.9 \pm 0.1	0.4 \pm 0.1	2.2
Ovar-5	0.9 \pm 0.1	0.4 \pm 0.1	2.2
CCD	0.9 \pm 0.1	0.1 \pm 0.01	9

To determine whether the L-plastin promoter was selectively more active in epithelial neoplastic (ovarian and bladder cancer) cell lines than in minimal deviation fibroblast cell line (CCD), we tested the LacZ gene expression levels in the Ovar-5, EJ, J82, Skov-3, and CCD cell lines after exposure to either the Ad-CMV-LacZ or the Ad-Lp-LacZ vectors. We then calculated the ratio of β -galactosidase levels in cells infected with the Ad-CMV-LacZ, divided by the β -galactosidase levels in cells infected by the Ad-Lp-LacZ vectors, as an index of the L-plastin promoter strength in established ovarian or bladder cancer cell lines as compared with the minimal deviation CCD fibroblast cell line.

As shown in Table 2, the amount of β -galactosidase in the EJ, J82, Skov-3, Ovar-5, and CCD cell lines after exposure to the Ad-Lp-LacZ vector was less than for the same cells exposed to the Ad-CMV-LacZ vector. This suggested that the Lp promoter was less strong in all of the cell lines than the CMV promoter, thereby decreasing the percentage of cells that scored positive for the vector transgene protein product under any given level of integrin or CAR receptor representation or function. A comparison of the amount of β -galactosidase in each cell line after exposure to the Ad-CMV-LacZ was divided by that for the Ad-Lp-LacZ vector. This ratio was 2 in all of the established cancer cell lines except for the CCD cell line, in which the ratio was 9. One possible explanation for this difference was that the CCD cell line supported the expression of the Lp promoter to a much lesser extent than the CMV promoter.

The relatively low LacZ gene expression in the CCD cell line exposed to the Ad-Lp-LacZ vector is not attributable to the low infectivity by the Ad-Lp-LacZ vector, because, as shown in Tables 1

and 2 and in a previous publication from our laboratory (7), >70% of the CCD cells were positive for β -galactosidase after exposure of these cells to the Ad-CMV-LacZ vector, indicating that the CCD cells are infectible by adenoviral vectors. The ratio of β -galactosidase levels in Ad-CMV-LacZ-infected cells divided by the β -galactosidase levels in Ad-Lp-LacZ-infected cells was much higher in CCD than in cell lines derived from bladder cancer and ovarian cancer. These data suggest that the L-plastin promoter is much more active in epithelial neoplastic cell lines than in the CCD minimal deviation fibroblast cell line.

Studies of the Effect of 5FU Released from Infected Cells on Noninfected Cells. To monitor the effect of 5FU released from infected cells on the noninfected cells, mixtures of Ad-CMV-CD or Ad-Lp-CD vector-infected and -noninfected cells were generated and then exposed to 5FC. The CD protein converts the nontoxic prodrug 5FC into the toxic chemical 5FU. Unphosphorylated 5FU can be released from cells infected with the CD vector and taken up by surrounding uninfected cells and can kill the uninfected cells. This is called the bystander effect. As shown in Fig. 1, when as few as 5% of the population of Ovar-5 cell lines or the CCD minimum deviation fibroblast cell line infected with Ad-CMV-CD (160 MOI) vectors were mixed with 95% of uninfected cells, the majority of the cells were killed when cells were exposed for 5 days to 5FC at a 500 μ M concentration (18% Ovar-5 and 29% CCD cells survived). This suggests that only a few of these cells need to be infected with the Ad-CMV-CD adenoviral vector to generate sufficient levels of 5FU *in vivo* in static cell culture to kill the vast majority of infected as well as uninfected tumor cells. The high percentage of cells killed at low infectivity *in vitro* is attributable partly to the fact that the medium was not changed, and therefore the cells were exposed continuously to a high level of 5FU, which continues to increase with time. In these conditions, the high levels of 5FU released from a few Ad-CMV-CD vector-infected cells could kill all of the uninfected cells.

When the cell lines were infected with the Ad-Lp-CD vector, incomplete cell death was seen even at the highest MOI tested with the CCD human minimal deviation cell line. In contrast, almost all of the cells were eradicated at the highest (160) MOI when similar experiments were carried out with the Ad-Lp-CD vector in the Ovar-5 cell line (see the data in Fig. 1, A and C, 160 MOI). The

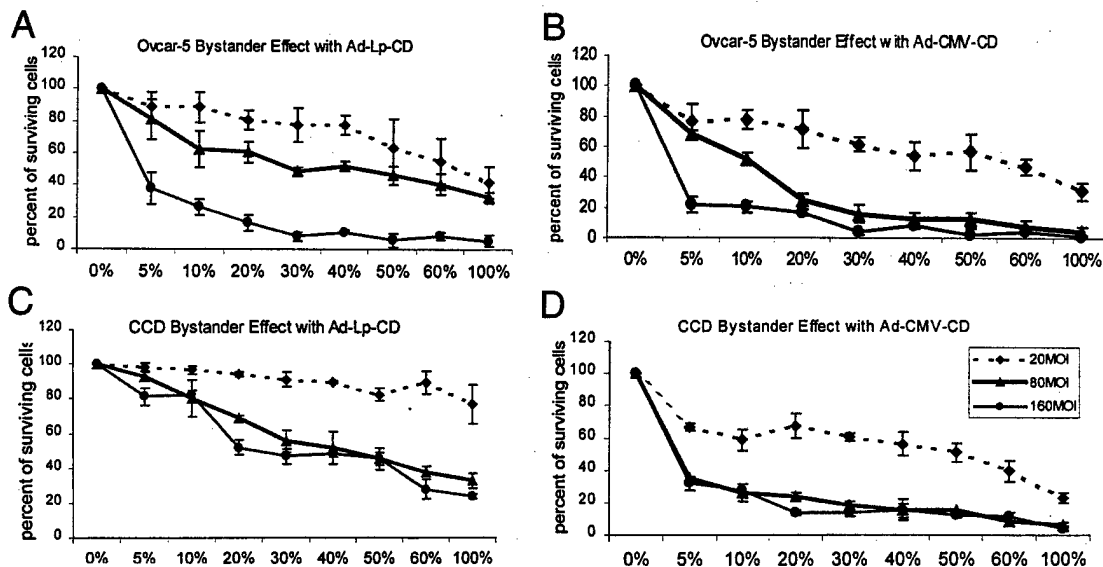


Fig. 1. Toxicity of vectors at varying levels of infected cells. Ovar-5 (A and B) or CCD (C and D) cell lines were infected at varying MOI (20, 80, and 160) using the Ad-CMV-CD (B and D) or Ad-Lp-CD (A and C) adenoviral vectors. The infected cells and noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells. Then cells were seeded in six-well plates and incubated for 5 days in 500 μ M 5FC. Then the cells were trypsinized, and surviving cells were counted by trypan blue exclusion.

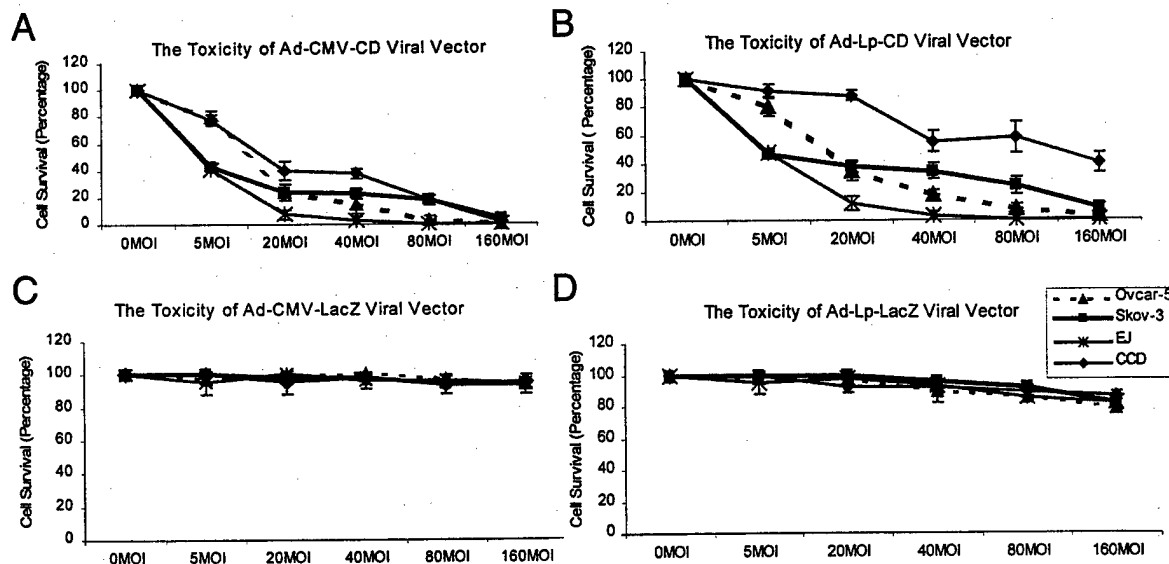


Fig. 2. Study of the toxicity of the control Lac-Z vector versus the CD vector. Cells (2×10^5) were infected at 0, 5, 20, 40, 80 and 160 MOI of vector with Ad-CMV-CD (A), Ad-Lp-CD (B), Ad-CMV-LacZ (C), Ad-Lp-LacZ (D) vectors for 90 min. Then cells were seeded in six-well plates in duplicate and incubated in 500 μ M 5FC for 5 days. The percentage of surviving cells was counted by trypan blue exclusion.

difference in the survival of cells between the CCD and Ovar-5 cells when exposed to the Ad-Lp-CD vector and 5FC was statistically significantly different at the $P < 0.001$ level. (An analysis of variance was used to determine whether the percentage of surviving cells was statistically significantly different when the Ad-CMV-CD or Ad-Lp-CD vectors were used to infect either the CCD or the Ovar cell lines.) This difference could be attributable to differences in the infectivity of the CCD cell line, the sensitivity of this cell line to 5FU, or a difference in the expression of the Lp-driven CD transcription units in the Ovar-5 or CCD cell lines. As shown in Fig. 1D, when the CCD cell line was exposed to 160 MOI of the Ad-CMV-CD vector and 5FC, complete killing of the CCD cells occurred at 100% infection. There were no differences between the cell kill in the Ovar-5 and CCD cell lines with the Ad-CMV-CD vector (compare the survival data at 160 MOI at 100% infection in Fig. 1, B and D). Therefore, the differences seen in A and C in the cell survival of the Ovar-5 and CCD cell lines after exposure to the Ad-Lp-CD vector are not attributable to differences between the CCD and Ovar-5 cell lines with respect to infectivity by the virus or sensitivity to 5FU, because complete killing is seen with the Ad-CMV-CD vector with the CCD cell line.

This suggests that the L-plastin promoter is less active in the CCD minimal deviation fibroblast cell line than in the established tumor cell line Ovar-5. The high levels of cell-killing at low infectivity that were seen *in vitro* with the Ad-Lp-CD vector will probably not be seen *in vivo* because of removal of the 5FU by blood flow and metabolic degradation.

5FU Sensitivity of Each Cell Line Expressed as IC_{50} . It is possible that the low-level cell death of the CCD cell line could be attributable to intrinsic resistance to 5FU toxicity, which is greater than that seen in the Ovar-5 or other established cancer cell lines. To test this, the intrinsic sensitivity of each cell line to 5FU was measured by seeding 3×10^5 cells in T25 flasks in triplicate, which were incubated for 96 h at different 5FU concentrations. The IC_{50} generated for 5FU in the J82 cell line is 55 μ M, for the EJ cell line is 30 μ M, for the Ovar-5 cell line is 3 μ M, for the Skov-3 cell line is 22 μ M, and for the CCD cell line is 15 μ M. The IC_{50} generated for 5FU in the CCD cell line (15 μ M) is less than that of several of the epithelial neoplastic cell lines (EJ, J82, and Skov-3), suggesting that the CCD cell line is as

sensitive to 5FU as the epithelial cancer cell lines. Thus, the low sensitivity of the CCD fibroblast cell line to the effect of the Ad-Lp-CD vector/5FC treatment is not attributable to a high level of resistance to 5FU, but rather to low levels of the protein product of the transcription units driven by the L-plastin promoter in the CCD cell line.

Study of 5FC Toxicity of the Adenoviral Vectors Carrying the CD Transcription Units. To test how much of the toxicity of the Ad-Lp-CD/5FU treatment was attributable to the toxicity of the vector backbone and how much was due to the protein produced by the CD transcription unit, the cell lines were infected with the Ad-CMV-CD, Ad-CMV-LacZ, Ad-Lp-CD, or Ad-Lp-LacZ vectors at different MOI. After this, the cell lines were incubated in medium supplemented with 500 μ M 5FC for 5 days. As shown in Fig. 2, no significant toxicity was seen with any of the cell lines when the backbone vector, Ad-CMV-LacZ, and Ad-Lp-LacZ were used (see Fig. 2, C and D). In contrast, when the cell lines were exposed to the Ad-Lp-CD or the Ad-CMV-CD vectors, nearly 100% killing of the cell lines after exposure to the vector and to 5FC was seen in all cell lines with both vectors, with the exception of the example of the CCD cell line after exposure to the Ad-Lp-CD vector and 5FC. The cell killing for CCD after exposure to the Ad-Lp-CD versus the Ad-CMV-CD vectors and 5FC (see Fig. 2, A and B) is statistically significantly different at the $P < 0.01$ level by the *t* test. No statistically significant differences were seen in any of the established tumor cell lines with respect to cell survival. This indicates that the toxicity seen in Fig. 2 after exposure to the Ad-Lp-CD or Ad-CMV-CD vectors is not attributable to the adenoviral backbone but to the action of the CD protein and 5FC. The $<100\%$ cell kill in the example of the CCD after exposure to the Ad-Lp-CD vector and 5FC is most probably attributable to the lower level of transcriptional activation of the CD gene by the Lp versus the CMV promoter, as explained above. Thus, the toxicity seen in these experiments was not attributable to the viral backbone, but to the effect of the CD transcription units on the conversion of 5FC to 5FU. In addition, the 5FC toxicity generated by incubation of the Ad-Lp-CD transcription units in bladder cancer or ovarian cancer cell lines is statistically significantly higher than that seen in the CCD cell line.

Table 3 Percentage of cells in explant cultures of ovarian cancer cells and normal peritoneal cells which score positive for β -galactosidase after exposure to the Ad-CMV-LacZ and the Ad-Lp-LacZ vectors

Samples of primary tumor, metastatic tumor, and normal peritoneum were cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation, and the resulting cells were cultured in RPMI 1640 with 10% NBCS. All experiments were performed at 90% confluence. Samples of ascites were divided into the T25 flasks directly and washed to remove debris after cell attachment. Cells were infected in the flasks for 90 min, and after 48 h of incubation, the positive cells were measured by X-Gal staining or FACS.

		Ascites	Primary tumor	Metastatic tumor	Normal peritoneum
Ad-CMV- β gal	X-Gal	50–80%	50–90%	45–85%	60–80%
	FACS	95%	94%	94%	
Ad-LP- β gal	X-Gal	10–35%	15–60%	15–45%	1–4%
	FACS	39%	83%	38%	
CMV/LP ratio	FACS	3/1	1/1	3/1	20–60/1

Percentage of Cells Detectable Positive for LacZ Expression in Primary Monolayer Cultures of Samples Obtained at Surgery from Normal Peritoneum and Metastatic Implants of Ovarian Cancer after Exposure to the Ad-Lp-LacZ or Ad-CMV-LacZ Vectors. Samples of metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy. The tumor was cut into small pieces and then digested with collagenase to disaggregate the tissue. The resulting cells were then cultured in RPMI 1640 supplemented with 10% NBCS. After culture, the cells were exposed at a MOI of 20 to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors in T-flasks for 90 min. After 48 h of incubation, the percentage of β -galactosidase-positive cells was measured by X-Gal staining or FACS (28). A ratio of β -galactosidase-positive cells with the two vectors was generated by dividing the percentage of cells that were detectable as positive for β -galactosidase by FACS after exposure to the Ad-CMV-LacZ vector by the percentage of cells detectable as positive for β -galactosidase by FACS after exposure to the Ad-Lp-LacZ vector. As shown in Table 3, this ratio was at least 20–60-fold higher in the normal peritoneal cells than with any of the samples derived from ovarian cancer cells. These results indicate that the normal peritoneal cells are less able to support the expression of transgenes driven by the L-plastin promoter than are the ovarian cancer cells.

Table 4 Cytotoxicity in monolayer culture of normal peritoneum and ovarian cancer cells after expression to Ad-Lp-CD and Ad-CMV-CD vectors and 5FC (percentage of cells killed)

In Ad-CMV-CD- and Ad-Lp-CD-infected samples, 500 μ M 5-FC were added and incubated for 5 days, then the percentage of cells killed was estimated by comparing the percentage of cells which had died in the infected and uninfected control flasks.

	Ad-CMV-CD	Ad-Lp-CD
Ascites	98%	85%
Metastatic tumor	85%	70%
Primary tumor	90%	75%
Normal peritoneum	95%	10%

Cytotoxicity after Exposure of the Monolayer Cell Cultures of Normal Peritoneum and Ovarian Cancer from Surgical Specimens to the Ad-CMV-CD and Ad-Lp-CD Vectors. Samples of primary tumor, metastatic tumor, and normal peritoneum were collected from 16 ovarian cancer patients, and samples were prepared by the same methods as described previously. As shown in Table 4, when the cells were infected with the CD vectors and incubated for 5 days in the presence of 500 μ M 5FC in T25 flasks, the majority of the cells in the explant cultures of primary ovarian cancer, metastatic ovarian cancer, and ovarian cancer in ascites were killed by the Ad-CMV-CD or the Ad-Lp-CD vectors and 5FC. In contrast with the results in the ovarian cancer cells, in which the cell death with the Ad-CMV-CD and Ad-Lp-CD vectors was roughly the same, in the case of the biopsies of normal peritoneum, the cell death with the Ad-Lp-CD vector was only one-tenth of that seen with the Ad-CMV-CD vector. This indicates that the expression of the L-plastin promoter-driven CD gene is much lower in the peritoneum than in the ovarian cancer cells.

Studies of LacZ Vectors in Organ Cultures of Normal Ovary. Samples of ovarian cancer and normal ovary tissues were cut into small pieces then inoculated in organ culture for 24–48 h and infected with either the Ad-CMV-LacZ or the Ad-Lp-LacZ vectors for 90 min. Then fresh medium was added, and the tissues were incubated for 48 h and then processed to the slide sections for study by the X-Gal staining reaction. The organ culture differs from the monolayer culture in that the organ culture is a three-dimensional array of cells. As shown in Fig. 3, there is a much stronger blue staining in the outer edges of the cell mass in the organ cultures of normal ovarian tissue with the Ad-CMV-LacZ vector, middle panel, than with the Ad-Lp-LacZ vector, right-hand panel. The results indicate that the CMV promoter is much more active in normal ovarian tissue than is the L-plastin promoter.

Killing Efficiency of Ovarian Cancer Tumor Cell Lines by 5FC/CD Vector System in Nude Mice. To test the efficacy of the Ad-Lp-CD replication incompetent vector system in a mouse human

Fig. 3. Ovarian organ cultures. Normal ovarian tissue was obtained from patients undergoing abdominal surgical procedures. The tissues were cut into small pieces and cultured in DMEM:Ham's F12 medium with 10% charcoal-stripped serum. Twenty-four to forty-eight h later, the tissues were infected with vectors for 90 min, washed with PBS, and then incubated for 48 h. Then the tissues were frozen in OCT and sectioned, after which the frozen sections were stained by the X-Gal reaction. Left, no vector; middle, Ad-CMV-LacZ vector; right, Ad-Lp-LacZ vector.

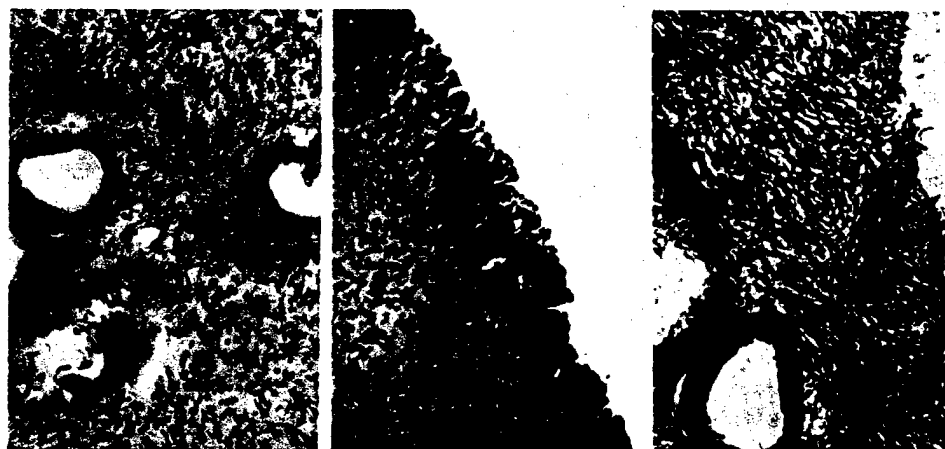


Table 5 Tumor growth in animals injected with adenoviral LP vectors (percentage of animals found to be positive for tumors)

The SCID mice were injected with 40 million Ovar-5 or Skov3 tumor cells, which had been infected previously *in vitro* with the Ad-Lp-LacZ vector or the Ad-Lp-CD vector. Starting on the second day, 500 mg/kg 5-FU was injected each day for 10 days. Animals were autopsied at 21 days after tumor cell injection, and the presence or absence of tumor nodules in the peritoneal cavity was assessed.

	Ad-Lp-LacZ-infected	Ad-Lp-CD-infected
Ovar-5 (100 MOI)	10/10 (100%)	0/10 (0%)
Skov3 (80 MOI)	5/5 (100%)	0/5 (0%)

tumor xenograft model, we first exposed the Skov-3 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 80 MOI or the Ovar-5 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 100 MOI by incubating the cells in the vector for 60 min. Then we injected 40 million of these *in vitro*-infected Ovar-5 vector infected cells into 10 nude mice or injected the *in vitro*-infected Skov-3 ovarian carcinoma cell line into 5 nude mice. One day after injecting the tumor cells, we initiated daily i.p. injections of 5FC into each of the animals to generate a daily peak of i.p. 5FC concentrations in the 500- μ M range. We carried on the daily i.p. 5FC injections for 10 days after the tumor injection. At 21 days after injection into the mice, we killed seven of the Ovar-5-injected mice and all five of the Skov3-injected mice and examined the peritoneal cavity for tumors. The remaining three Ovar-5 mice that were not killed at 21 days were killed at 50 days after tumor injection. As shown in Table 5, all of these animals were free of detectable tumor nodules, either at the gross morphological level or at the histopathological level.

In contrast, as shown in Table 5, all of the animals injected with the Ovar-5 *in vitro*-infected cell lines and all five of the animals injected with the Skov-3 *in vitro* tumor cells previously infected *in vitro* with

the control Ad-Lp-LacZ virus had detectable signs of tumor cell growth, either at the gross level or at the microscopic level. These data show that, in principal, it is possible to prevent engraftment of tumor cells in nude mice if all of the tumor cells are infected *in vitro* before i.p. injection of the cells with the replication-incompetent Ad-Lp-CD vector, and the animals are injected on a daily basis with the prodrug (5FC) which is converted into 5FU in the tumor cells.

To test the effect of administering the replication-incompetent Ad-Lp-CD and the Ad-CMV-CD vectors *in vivo* to preexisting s.c. nodules, we also tested the effect of intratumoral *in vivo* injection of established tumor nodules with the LacZ control vector, the CMV-CD vector, and Lp-CD adenoviral vector on the growth of the s.c. tumor nodules. As shown in Fig. 4, the tumors injected with the control Ad-CMV-LacZ vectors increased 3–4-fold after vector injection. In contrast, the size of both the Ovar-5 and the EJ cell tumor nodules injected with the Ad-CMV-CD or the Ad-Lp-CD vector was one-third to one-sixth of the size of the tumors injected with the Ad-CMV-LacZ vector. The growth of the Ovar-5 or EJ cancer cell lines after exposure to the Ad-CMV-LacZ vector was statistically significantly greater than the growth of the Ovar-5 or EJ cell lines after exposure to either the Ad-CMV-CD or the Ad-Lp-CD vectors, at the $P < 0.001$ level, by the *t* test of the ratios (two-tailed). There was no statistically significant difference in the growth of the Ovar-5 or the EJ cancer cell lines exposed to the Ad-CMV-CD *versus* the Ad-Lp-CD vectors.

To determine whether there was toxicity to the normal tissues, we studied histopathological sections of the tumor nodules and surrounding normal tissues by light microscopic examination after injection with the Ad-Lp-CD, Ad-CMV-CD, or Ad-CMV-LacZ vectors after exposure to 5FC. As seen in Fig. 5, *in vivo* injection of the Ad-Lp-CD

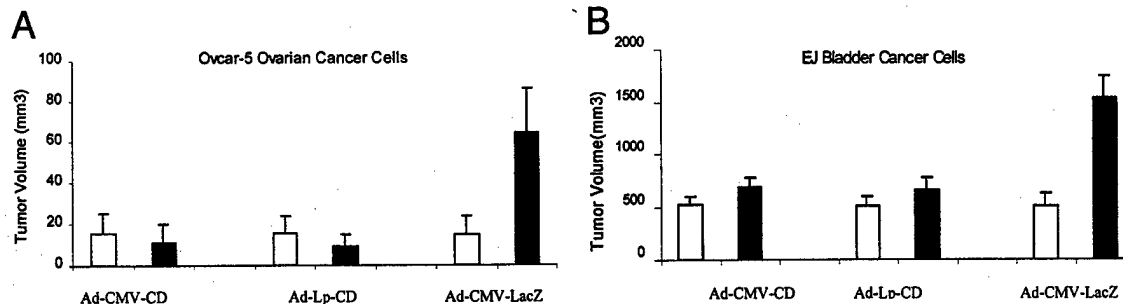
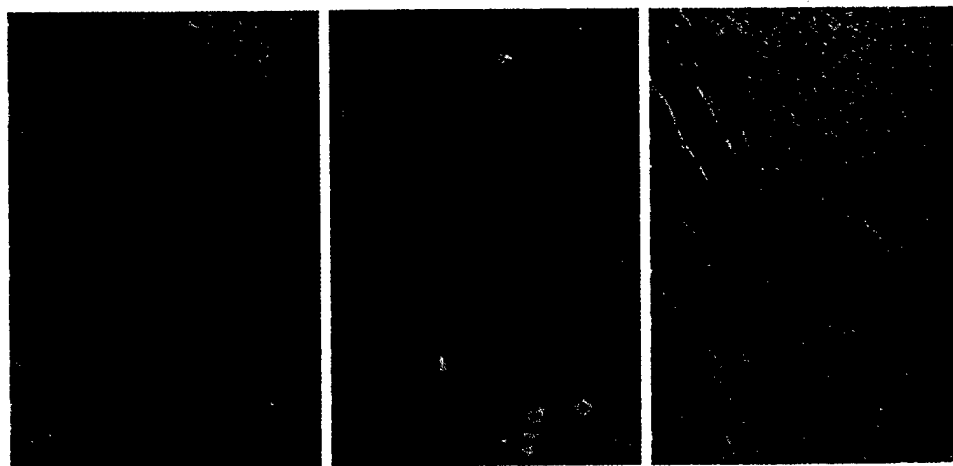


Fig. 4. Effect of *in vivo* injection of tumor nodules with adenoviral vectors. Ovar-5 (A) or EJ (B) cells (5×10^6) were injected s.c. into nude mice. After 3 weeks, the tumor nodules were measured. Then, 10^8 pfu of the Ad-CMV-CD, 10^8 pfu of the Ad-Lp-CD, or 10^8 pfu of the Ad-CMV-LacZ vectors were injected into each tumor nodule, and 500 mg/kg of 5FC was given i.p. once a day for 5 days. Seven days later, the tumor nodules were measured again. □ shows tumor volume before viral particles and 5FC treatment; ■ shows the tumor volume 7 days after exposure to viral particles and 5FC treatment.

Fig. 5. Vector toxicity to tumor cells and adjacent tissues. Ovar-5 cells (5×10^6) were injected s.c. After 3 weeks, 10^8 pfu of Ad-Lp-CD (right), 10^8 pfu of Ad-CMV-CD (left), or 10^8 pfu of Ad-CMV-LacZ (middle) vector were injected into each tumor nodule, and 500 mg/kg 5FC was given i.p. once a day for 5 days. Right (Ad-Lp-CD), most of the tumor cells are necrotic, whereas the adjacent muscle cells have a normal structure. Left (Ad-CMV-CD), after injection with the Ad-CMV-CD vector, the tumor cells are necrotic. Middle (Ad-CMV-LacZ), after injection of the Ad-CMV-LacZ vector, neither the muscle nor the tumor are necrotic.



vectors into the tumor nodules generated toxicity to the tumor cells (right). In Ad-CMV-CD *in vivo*-injected tumors, the tumor cells underwent necrosis (left). The toxicity to the tumor with the Ad-Lp-CD vectors was every bit as extensive in the tumor as that seen with the Ad-CMV-CD vector. This data shows that the toxic effect of the Ad-Lp-CD vector/5FC system is as great as that generated by the Ad-CMV-CD/5FC system, and the toxic effect of these two vectors is much greater than that seen with the Ad-CMV-LacZ vector.

DISCUSSION

A major limitation of the existing adenoviral vectors used for cancer gene therapy is the nonselective toxic action of these vectors. Attempts to render these vectors more selective for tumor cells and less toxic for normal cells has involved the use of tissue-specific transcriptional promoters to drive the therapeutic transcription units for these vectors. One of the limitations that have characterized these tissue-specific promoters is that the vectors carrying these tissue-specific therapeutic transcription units are usually less robust in their antitumor toxic action than nonselective viral transcriptional promoters.

We have reported the use of a tumor-specific rather than a tissue-specific transcriptional promoter for the regulation of an adenoviral therapeutic transcription unit. The L-plastin promoter was chosen because no normal tissue except for the mature leukocyte exhibits expression of the *L-plastin* gene. In contrast, most of the established cancer cell lines exhibit high levels of the expression of this gene. Experimental results published previously by our laboratory (7) have shown that a truncated L-plastin promoter retained its high activity within ovarian cancer cells, whereas it was relatively inactive in explants of normal peritoneal lining mesothelial cells. This data suggested that adenoviral vectors carrying therapeutic transcription units regulated by the L-plastin promoter might be useful in treating ovarian cancer.

When the L-plastin promoter is used to drive the expression of CD chemotherapy sensitization transcription unit in static cultures *in vitro*, only 50% of the cancer cells need to be infected to kill 100% of the epithelial neoplastic cells. In contrast, the percentage of cells that die in populations of CCD fibroblast cells is much lower, never reaching 100%. At all MOI tested, there are statistically significant different levels of cell death generated by exposure to the Ad-CMV-CD *versus* the Ad-Lp-CD vectors and 5FC ($P < 0.001$), presumably because of the lower levels of activity of the L-plastin promoter in the CCD cell line (see Fig. 1 and 2). Control experiments have shown that the CCD cell line is as infectible by the Ad-CMV-LacZ as are the epithelial cancer cell lines (see Table 1). This indicates that low infectivity is not responsible for the low sensitivity of the CCD cell line to the Ad-Lp-CD vector. In addition, the intrinsic sensitivity of the CCD to 5FU toxicity directly added to the culture is not lower than that seen in the epithelial neoplastic cell lines. Thus, it appears that the level of expression of the L-plastin-driven genes in the CCD cell line is lower than that seen in the ovarian and bladder cancer cell lines and this is responsible for the differential effect of the Ad-Lp-CD and Ad-CMV-CD vectors in the CCD *versus* the epithelial neoplastic cell lines.

Studies in primary normal mesothelial cells and primary cell cultures of ovarian cancer cells show that the ratio of cytotoxicity with CMV-driven CD adenoviral vectors: to Lp-driven CD adenoviral vectors is highest in normal peritoneum (ratio of 20–60) as compared with 3–5 times in ovarian cancer cells in malignant ascites or in primary or metastatic ovarian cancer. The use of the Ad-Lp-CD vector to infect ovarian cancer cell lines *in vitro* before their injection into the i.p. cavity of 5FC-injected nude mice results in a suppression of the

engraftment of these ovarian cancer cells, whereas no sign of suppression of tumor growth occurred when the ovarian cancer cell lines were infected with the Ad-Lp-LacZ or Ad-CMV-LacZ control vectors.

These data suggest that, in principle, the L-plastin-regulated CD transcription units may selectively sensitize ovarian cancer cell lines to the effects of 5FC without significantly sensitizing the normal peritoneal surface cells to the effects of this 5FC/Vector system.

Many obstacles that remain to be overcome are pointed up by this data. The first is that, for such vectors to work *in vivo* in patients, some method must be developed for conferring conditional replication competency on these Lp-CD vectors so that they may infect 100% of the tumor cells when administered to patients with existing tumor *in vivo*.

The data in Fig. 3 shows that the expression of the reporter gene is seen only on the surface of an organ culture of cells infected with a replication-incompetent vector. Therefore, our laboratory is studying, on a preclinical level, several different types of adenoviral vectors that exhibit replication competency that is selective for the regulatory environment of the tumor cell. Our design is to use the L-plastin promoter to drive the expression of the adenoviral *E1A* gene, which is necessary for viral replication, as well as the CD chemotherapy sensitization gene. Such vectors may be useful in the i.p. therapy of ovarian cancer.

REFERENCES

- Descamps, V., Duffour, M. T., Mathieu, M. C., Ferbadez, N., Cordier, L., Abina, M. A., Kremer, E., Perricaudet, M., and Haddada, H. Strategies for cancer gene therapy using adenoviral vectors. *J. Mol. Med.*, 74: 183–189, 1996.
- Koyama, F., Sawada, H., Hirao, T., Fujii, H., Hamada, H., and Nakano, H. Combined suicide gene therapy for human colon cancer cells using adenovirus-mediated transfer of *Escherichia coli* cytosine deaminase gene and *Escherichia coli* uracil phosphoribosyltransferase gene with 5-fluorocytosine. *Cancer Gene Ther.*, 7: 1015–1022, 2000.
- Xiang, J., Chen, Y., and Moyana, T. Combination immunotherapy for established tumors with engineered tumor vaccines and adenovirus-mediated gene transfer. *Cancer Gene Ther.*, 7: 1023–1033, 2000.
- Leavitt, J. Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. *Electrophoresis*, 15: 345–357, 1994.
- Lin, C. S., Chang, C. H., and Huynh, T. The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol.*, 1: 9–16, 1997.
- Park, T., Chen, Z. P., and Leavitt, J. Activation of the leukocyte plastin gene occurs in most human cancer cells. *Cancer Res.*, 54: 1775–1781, 1994.
- Chung, L., Peter, E. S., Crystal, R. G., Pizzorno, G., and Deisseroth, A. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Ther.*, 6: 99–106, 1999.
- Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J., and Crystal, R. G. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum. Gene Ther.*, 6: 1055–1063, 1995.
- Tong, X., Engehausen, D. G., Freund, C. T., Agoulnik, I., Guo, Z., Oehler, M. K., Kim, T. E., Hasenburger, A., and Kieback, D. G. The efficacy of adenovirus-mediated gene therapy of ovarian cancer is enhanced by using the cytomegalovirus promoter. *Anticancer Res.*, 18: 719–725, 1998.
- Graham, F. L., and Prevec, L. Manipulation of adenovirus vectors. In: E. J. Murray (ed.), *Methods in Molecular Biology*, Vol. 7, pp. 109–128. Clifton, NJ: The Human Press Inc., 1991.
- Neering, S. J., Hardy, S. F., Minamoto, D., Spratt, S. K., and Jordan, C. T. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood*, 88: 1147–1155, 1996.
- Couffignal, T., Kearney, M., Sullivan, A., Silver, M., and Isner, J. M. Histochemical staining following *LacZ* gene transfer underestimates transfection efficiency. *Hum. Gene Ther.*, 8: 929–934, 1997.
- Theodore, L., Rehemtulla, A., Emily, Y., Trosko, J. E., and Stetson, P. L. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-fluorocytosine. *Cancer Res.*, 58: 2588–2593, 1998.
- Kianmanesh, A. R., Perrin, H., Panis, Y., Houssin, D., and Klatzmann, D. A. "Distant" bystander effect of suicide gene therapy: regression of nontransduced tumors together with a distant transduced tumor. *Hum. Gene Ther.*, 8: 1807–1814, 1997.
- Ramesh, R., Marrogi, A. J., Munshi, A., and Freeman, S. M. Potentiation of the bystander effect by immunization combined with suicide gene therapy. *Adv. Exp. Med. Biol.*, 451: 125–131, 1998.
- Parsels, L. A., Kowalski, M. P., Wagner, L. M., Parsels, J. D., Maley, F., and Maybaum, J. *Escherichia coli* thymidylate synthase expression protects human cells

- from the cytotoxic effects of 5-fluorodeoxyuridine more effectively than human thymidylate synthase overexpression. *Cancer Gene Ther.*, 7: 1179–1187, 2000.
17. Sapi, E., Flick, M. B., and Rodov, S. Expression of CSF-1 and CSF-1 receptor by normal lactating mammary epithelial cells. *J. Soc. Gynecol. Investig.*, 5: 94–101, 1998.
 18. Kelly, L., Molpus, D. K., Leonard, A., and Hasan, T., Characterization of a xenograft model of human ovarian carcinoma which produces intraperitoneal carcinomatosis and metastases in mice. *Int. J. Cancer*, 67: 588–595, 1996.
 19. Hwang, E. S., Kim, J. S., You, E. H., Lee, S. H., and Lee, J. H. Intraperitoneal gene therapy with adenoviral-mediated *p53* tumor suppressor gene for ovarian cancer model in nude mouse. *Cancer Gene Ther.*, 6: 172–178, 1999.
 20. Pang, S. Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. *Cancer Gene Ther.*, 7: 991–996, 2000.
 21. Martin, D. S., Stolfi, R. L., Sawyer, R. C., and Yang, C. W. Therapeutic utility of utilizing low doses of *N*-(phosphonacetyl)-L-aspartic acid in combination with 5-fluorouracil: a murine study with clinical relevance. *Cancer Res.*, 43: 2317–2321, 1983.
 22. Bergelson, J. B., Krithivas, A., Celi, L., Wickham, T., Crowell, R. L., and Finberg, R. W. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J. Virol.*, 72: 415–419, 1998.
 23. Bergelson, J. M., Cunningham, J. A., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* (Wash. DC), 275: 1320–1323, 1997.
 24. Roelvink, P. W., Lizonova, A., Lee, J. M., Li, Y., Kovesdi, I., and Wickham, T. J. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J. Virol.*, 72: 7909–7915, 1998.
 25. Garcia-Sanchez, F., Fu, S. Q., Nanakorn, T., Leffert, J. J., Cote, R., Crystal, R., and Deisseroth, A. Cytosine deaminase adenoviral vector and 5-fluorocytosine selectively reduce breast cancer cells 1 million-fold when they contaminate hematopoietic cells: a potential purging method for autologous transplantation. *Blood*, 92: 672–682, 1998.
 26. Wickham, T. J., Mathias, P., Cheres, D. A., and Nemerow, G. R. Integrins $\alpha v \beta 3$ and $\alpha v \beta 5$ promote adenovirus internalization but not virus attachment. *Cell*, 73: 309–319, 1994.
 27. Wickham, T. J., Filardo, E. J., Cheres, D. A., and Nemerow, G. R. Integrin $\alpha v \beta 5$ selectively promotes adenovirus mediated cell membrane permeabilization. *J. Cell Biol.*, 127: 257–264, 1993.
 28. Roederer, M., Fiering, S., and Herzenberg, L. A. FACS-gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. *Methods*, 2: 248–260, 1991.

Adenoviral Vectors with E1A Regulated by Tumor-Specific Promoters Are Selectively Cytolytic for Breast Cancer and Melanoma

Lixin Zhang,^{1,2} Hakan Akbulut,¹ Yucheng Tang,¹ Xueyuan Peng,² Giuseppe Pizzorno,² Eva Sapi,² Sebastian Manegold,² and Albert Deisseroth^{1,*}

¹Genetic Therapy Program of the Sidney Kimmel Cancer Center, San Diego, California 92121, USA

²Yale University School of Medicine, New Haven, Connecticut 06520, USA

*To whom correspondence and reprint requests should be addressed. Fax: (858) 587-8873. E-mail: adeisseroth@skcc.org.

We have previously demonstrated that a truncated form of the L-plastin promoter can confer tumor-specific patterns of expression on replication-incompetent adenoviral vector reporter and therapeutic transcription units. In this report, a 2.5-kb truncated version of the L-plastin promoter was placed 5' to the E1A gene of a wild-type adenovirus. The vector generated (Ad-Lp-E1A) was directly cytotoxic to established breast and ovarian cancer cell lines and to primary explant cultures derived from ovarian cancer, but was not cytotoxic to explant cultures of normal mammary epithelial cells. This vector was not cytotoxic to cell lines in which the L-plastin E1A transcription unit was not expressed, whereas the same cell lines were sensitive to the cytotoxic effect of a replication-competent adenoviral vector in which the cytomegalovirus (CMV) promoter drove E1A expression. When the tyrosinase promoter/enhancer was placed 5' to the E1A gene in the adenoviral backbone, the resulting vector (Ad-Tyr-E1A) was selectively toxic to melanoma cells and one percent as toxic to explants of ovarian cancer cells as the Ad-Lp-E1A vector. Injection of these vectors (Ad-Lp-E1A and Ad-Tyr-E1A) into nodules derived from the MCF-7 and MDA-MB-468 human breast cancer cell lines and the TF-2 human melanoma cell line, respectively, which were growing subcutaneously in severe combined immunodeficiency (SCID) mice, induced regression of these tumors. Such vectors may therefore be useful in cancer treatment.

Key Words: adenovirus, breast cancer, ovarian cancer, melanoma, E1A, L-plastin

INTRODUCTION

Recombinant adenoviral vectors have been widely used in preclinical models for *in vitro* and *in vivo* gene transfer. Adenoviral vector-mediated therapeutic gene expression has been achieved in a broad spectrum of eukaryotic cells and is independent of cell replication [1,2]. In addition, the E1 gene-deleted, replication-defective adenoviral vectors can accommodate large DNA inserts. Tissue-specific promoters have been used to regulate the expression of therapeutic transgenes in these vectors to restrict the toxicity of the treatment to the tissue of origin of the cancer [3]. One of the problems is the low percentage of cells in a tumor nodule that eventually becomes infected by replication-incompetent adenoviral vectors. Tissue-specific promoters or enhancers have also been used to regulate the expression of viral genes that are necessary to the replication of adenoviral vectors, as well as herpes simplex recombinant vectors to increase the number of cancer cells

infected by the vector [4,5]. In this context, the minimal promoter/enhancer from the prostate-specific antigen (PSA) gene has been used to drive E1A expression and to create an adenovirus, designated CN706, that selectively replicates in PSA-positive cells [4]. A similar strategy using the albumin promoter has been used to develop a herpes simplex virus that selectively replicates in hepatoma cells [5]. Genetic complementation between mutations introduced into the E1B gene of the adenoviral vector and mutations in tumor suppressor genes in the tumor cells have been used to create adenoviral vectors that are conditionally replication-competent in the cancer cells but not in the normal tissues of the host [6].

We have been studying the feasibility of using a truncated form of the L-plastin promoter to create adenoviral vectors that selectively replicate within tumor cells and, therefore, are selectively toxic to these cells. The plastins constitute a family of human actin-binding proteins (isoforms) that are abundantly expressed in all normal

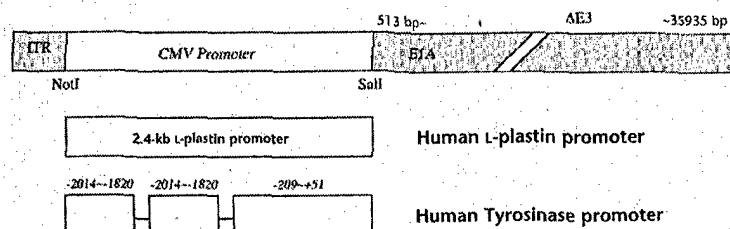


FIG. 1. Organization of conditionally replication-competent adenoviral vectors. The 2.5-kb L-plastin promoter or the human tyrosinase promoter and tissue-specific enhancer were inserted 5' to the E1A and E1B genes. The regulatory elements left in the promoter in the adenoviral vectors are identical to those reported by Rodríguez *et al.* [4]. The viral packaging signal, which overlaps the E1A enhancer, has also been left in the area 5' to the E1A transcription unit. Finally, it should be emphasized that both the E1A and E1B genes have been left in the vector.

replicating mammalian cells. One isoform, L-plastin, is constitutively expressed at high levels in mature hematopoietic cell types but is expressed in no other normal tissue. L-Plastin is, however, constitutively synthesized in most types of malignant human cells, suggesting that its expression is induced during tumorigenesis. L-Plastin expression is especially high in cancers that arise from estrogen-dependent tissues [7–10]. To test the feasibility of conferring tumor-specific conditional replication competency on the wild-type adenovirus, we have placed a 2.5-kb truncated form of the L-plastin promoter (Fig. 1) 5' to the E1A gene (Ad-Lp-E1A). For comparison, we have also placed either the CMV promoter (Ad-CMV-E1A) or the tyrosinase promoter/enhancer (Fig. 1) 5' to the E1A gene in a wild-type adenovirus. Tyrosinase, which is the product of the albino locus and is a pigment cell-restricted enzyme that catalyzes the rate-limiting step in melanin synthesis, is highly expressed in melanoma cells. The tyrosinase promoter/enhancer cassette used for this purpose has been studied in human and mouse cells [11–13] for its ability to govern the expression of heterologous genes in the adenoviral vector.

The Ad-Lp-E1A vector is toxic to the L-plastin-positive MCF-7 and MDA-MB-468 established human breast cancer cell lines, as well as to explants of human ovarian cancer cells *in vitro*, but is not toxic to explant cultures of normal mammary epithelial cells. When injected intratumorally, the Ad-Lp-E1A vector suppresses the growth of L-plastin-positive breast cancer cells in severe combined immunodeficiency (SCID) mice. We also demonstrate that the vector that contains the tyrosinase promoter/enhancer cassette driving the E1A gene (Ad-Tyr-E1A) suppresses the growth of the TF-2 human melanoma

cell line in SCID mice. The results demonstrate that the Ad-Lp-E1A vector in which E1A gene expression is regulated by a truncated L-plastin promoter is 100 times as toxic to ovarian cancer cells as the Ad-Tyr-E1A vector, which itself is toxic to melanoma cell lines. Our results also suggested that a correlation exists between the ability of cancer cell lines to support the expression of the E1A gene following exposure to the Ad-Lp-E1A vector, and the degree of detachment and lysis of the exposed cancer cells.

RESULTS

Construction of Conditionally Replication-Competent Adenoviral Vectors

To construct replication-competent adenoviral vectors, we inserted the 2.5-kb L-plastin promoter *ScaI* fragment or the cytomegalovirus (CMV) promoter or the tyrosinase promoter/enhancer 5' to the E1A gene of the wild-type adenovirus (Fig. 1) according to the reported method [14]. After adenoviral vector particles were generated in HEK 293 cells, the vector DNA was purified. The presence and structure of the promoter in each adenoviral vector was confirmed by PCR, by restriction enzyme analysis, and by DNA sequencing of the promoter in the AD5 sequence.

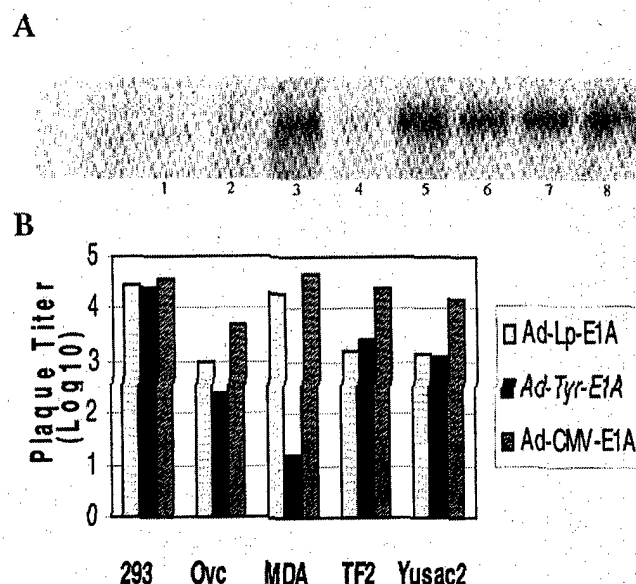
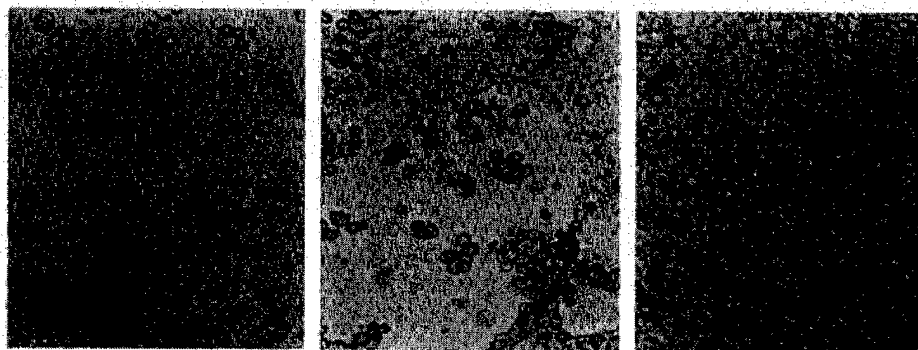


FIG. 3. Study of the sensitivity of breast cancer cells to the cytotoxic effect of the Ad-Lp-E1A vector. The MDA-MB-468 breast cancer cell line was exposed to the Ad-Lp-E1A vector at an MOI of 100 (left) and at an MOI of 10 (middle). The cells in the right panel were not exposed to vector. The pictures were taken 9 days after infection.



Western Blot Analysis of E1A Expression

E1A protein production in cells infected with Ad-Lp-E1A (Fig. 2A, lanes 1–4) and in those infected with Ad-CMV-E1A, as defined in Fig. 1, (Fig. 2A, lanes 5–8) was determined by western blot analysis. The Ad-CMV-E1A adenoviral vector produced considerable E1A protein in the Yusac2 and TF2 human melanoma cell lines and in the MDA-MB-468 human breast cancer cell line (Fig. 2A, lanes 5–7). Although the Ad-Lp-E1A produced a high level of E1A protein in the MDA-MB-468 established breast cancer cell line (Fig. 2A, lane 3), it produced a much lower level of expression in subconfluent explant cultures of normal human mammary epithelial cells (Fig. 2A, lane 4). These data show that the L-plastin promoter can drive the expression of the E1A gene in breast cancer cells but not in explant cultures of normal human mammary epithelial cells, suggesting a tumor-specific pattern of expression. The L-plastin promoter in Ad-Lp-E1A produces a much lower level of E1A expression in the TF-2 human melanoma cell line (Fig. 2A, lane 2) than in the MDA-MB breast cancer cell line. This suggests a tissue-specific pattern of expression of these vectors as well. The fact that the Ad-CMV-E1A vector produced high levels of E1A expression in all of the cell lines tested (Fig. 2A, lanes 5–8), even the ones in which the Ad-Lp-E1A and Ad-Tyr-E1A vectors produced only very low or undetectable levels of E1A expression, suggests that the low levels of expression in certain cell lines following exposure to the Ad-Lp-E1A and Ad-Tyr-E1A vectors was not due to difficulty in infecting the target cell lines but due to the low strength of the promoters in these cell lines.

The replication of the viral vectors Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A in different cell lines (293, Ovar-5, MDA-MB-486, TF2, and Yusac2) was determined by assaying the replicated virus yield of the

vectors in those cell lines (Fig. 2B). The replication of the Ad-Lp-E1A and Ad-CMV-E1A vectors is equivalent in the MDA-MB-468 human breast cancer cell line. The Ad-Tyr-E1A vector can replicate in the TF2 and Yusac2 human melanoma cell lines, but replicates much less in the MDA-MB-468 breast cancer cell line. These data suggest that the replication of these vectors will exhibit tissue and tumor-specific patterns, in agreement with the E1A expression data presented in Fig. 2A.

Cytolysis Assays in Cell Lines and Explant Cultures of Tumor Cells Following Exposure to Viral Vectors

To test if the replication of the Ad-Lp-E1A and Ad-Tyr-E1A vectors results in a direct cytotoxic effect, these vectors were analyzed for their ability to generate rounding up and detachment of established human cancer cell lines and explant cultures of human tumor cells derived from excised ovarian cancer tissue from patients. The MDA-MB-486 human breast cancer cell line, which has been shown in our laboratory to support the expression

Explants of Normal Mammary Epithelial Cells

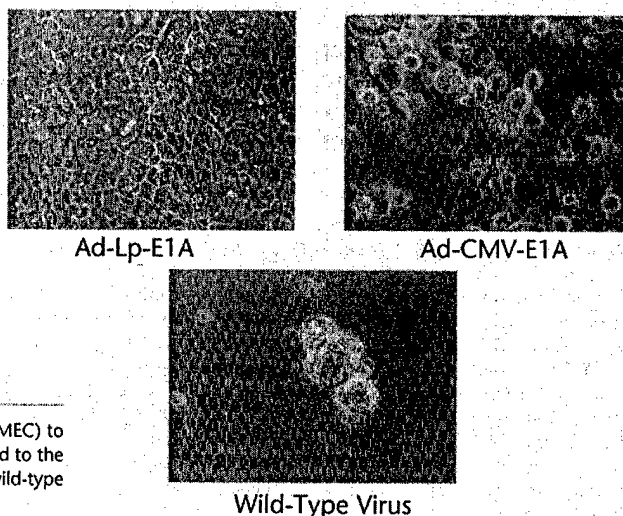


FIG. 4. Study of the sensitivity of normal human mammary epithelial cells (HMEC) to the cytotoxic effect of the Ad-Lp-E1A vector. Normal HMEC cells were exposed to the Ad-Lp-E1A vector (top left), to the Ad-CMV-E1A vector (top right), and to wild-type adenovirus (bottom) at an MOI of 10 for 9 days.

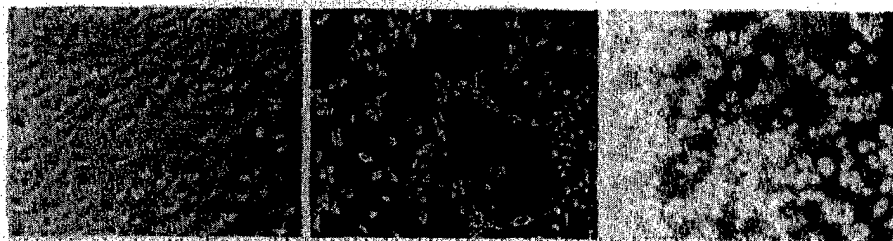


FIG. 5. Study of the sensitivity of explant cultures of human ovarian cancer cells to the cytotoxic effect of the Ad-Lp-E1A vector 9 days after infection. Explant cultures of human ovarian cancer cells were exposed to the Ad-Lp-E1A vector at MOIs of 1 and 10 (middle and right, respectively) or were not exposed to the vector (left).

of genes regulated by the L-plastin promoter [7,15] and has been shown in this work to support the expression of the E1A gene when exposed to the Ad-Lp-E1A vector (Fig. 2A, lane 3), was used to test the lytic effect of the Ad-Lp-E1A vector *in vitro*. The MDA-MB-468 cells were infected with Ad-Lp-E1A at different MOIs. Complete lysis of the cells was observed 9 days after infection at an MOI of 10 (Fig. 3 middle). Another breast cancer cell line, MCF-7, produced the same result (data not shown).

Normal human mammary epithelial cells, which showed only very low expression levels of the E1A gene following exposure to the Ad-Lp-E1A vector (Fig. 2A, lane 4) but showed E1A expression following exposure to the Ad-CMV-E1A adenoviral vector (Fig. 2A, lane 8), were exposed to the Ad-Lp-E1A and the Ad-CMV-E1A vectors and to the wild-type adenovirus at an MOI of 10. A rounding up and lifting off of the cells was observed following exposure of the normal human mammary epithelial cells to the Ad-CMV-E1A vector or to the wild-type adenovirus after 9 days (Fig. 4). In this experiment, no lysis was seen following exposure of the normal human mammary epithelial cells to the Ad-Lp-E1A vector (Fig. 4). Thus, the results in Figs. 2–4 show that there is a correlation between the expression of the E1A gene, the replication of the vector, and the lysis of the cell lines following exposure to the Ad-Lp-E1A vector. These results suggest that Ad-Lp-E1A vector can replicate only in cells that support the expression of genes regulated by the L-plastin promoter.

To test for a direct cytolytic effect of the Ad-Lp-E1A vector in human ovarian carcinoma cells, explants of ovarian cancer cells excised from patients were cultured as monolayers and then exposed to the Ad-Lp-E1A vector. At MOIs of 1 and 10, a remarkable lytic effect was observed in the explant cultures of ovarian carcinoma (Fig. 5). Then, the effect of the Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A

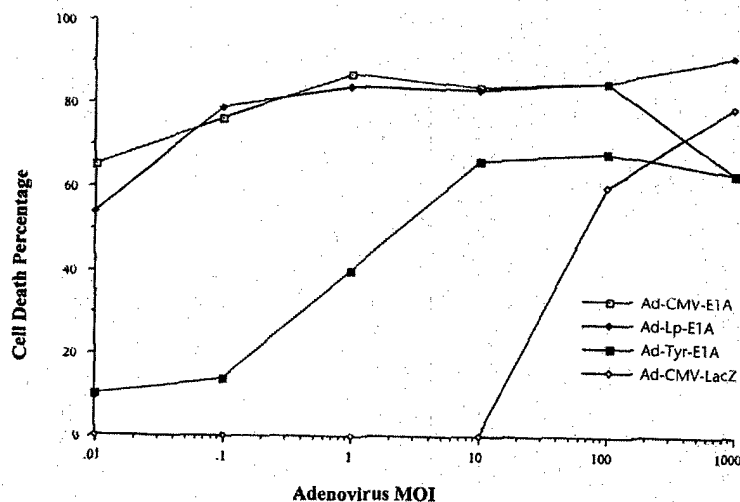
vectors and the negative control Ad-CMV-LacZ adenoviral vector on explant cultures of human ovarian carcinoma cells from a patient sample was tested at different MOIs (Fig. 6). The difference in the IC₅₀ between the Ad-Lp-E1A and Ad-Tyr-E1A vectors is about 100-fold (Fig. 6). The difference in the IC₅₀ between the Ad-Lp-E1A and the Ad-CMV-LacZ vectors was 1000-fold. In addition, the data show that the cytolytic effect of the Ad-Lp-E1A vector is equivalent to that of the Ad-CMV-E1A vector.

When we infected the TF-2 human melanoma cell line with the Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A vectors, as well as with the wild-type adenovirus, the cultures exposed to the Ad-CMV-E1A and Ad-Tyr-E1A vectors, as well as the wild-type adenovirus 5, showed lytic effects. In contrast, cultures of the TF-2 human melanoma cell line exposed to the Ad-Lp-E1A vector did not show detectable cytotoxicity (Fig. 7). These data show that the toxic effect of the Ad-Lp-E1A vector is much lower in the TF2 human melanoma cell line than in the ovarian or breast cancer cell lines. This suggests that the L-plastin promoter, when placed 5' to the E1A gene in the adenoviral vector, can act as a tissue-specific, as well as tumor-specific, promoter.

Treatment of Human Breast Cancer Xenograft with the Ad-Lp-E1A Vector and of Human Melanoma Xenograft with the Ad-Tyr-E1A Vector

To evaluate the therapeutic efficacy of the Ad-Lp-E1A vector *in vivo*, MCF-7 and MDA-MB-468 human breast cancer

FIG. 6. Study of the sensitivity of explant cultures of ovarian cancer cells to adenoviral vectors. Monolayer explant cultures of ovarian cancer cells were exposed to varying MOIs of the Ad-Lp-E1A vector, of the Ad-CMV-E1A vector, of the Ad-Tyr-E1A vectors, and of the replication-incompetent Ad-CMV-LacZ negative control vector. The percentage of cells killed was measured by the MTT assay [15] 7 days after infection. The percentage of cells killed is calculated as the ratio of the absorbance at 490 nm in the test culture to that of the culture without vector added. All data are the result of triplicate assays, and the standard deviation is less than 15%.



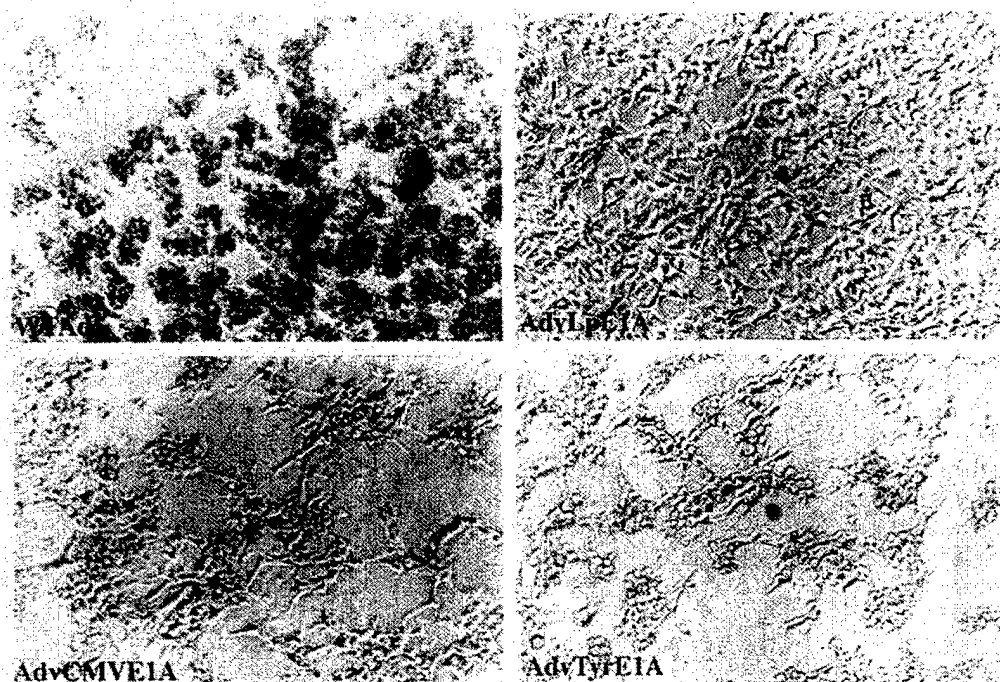
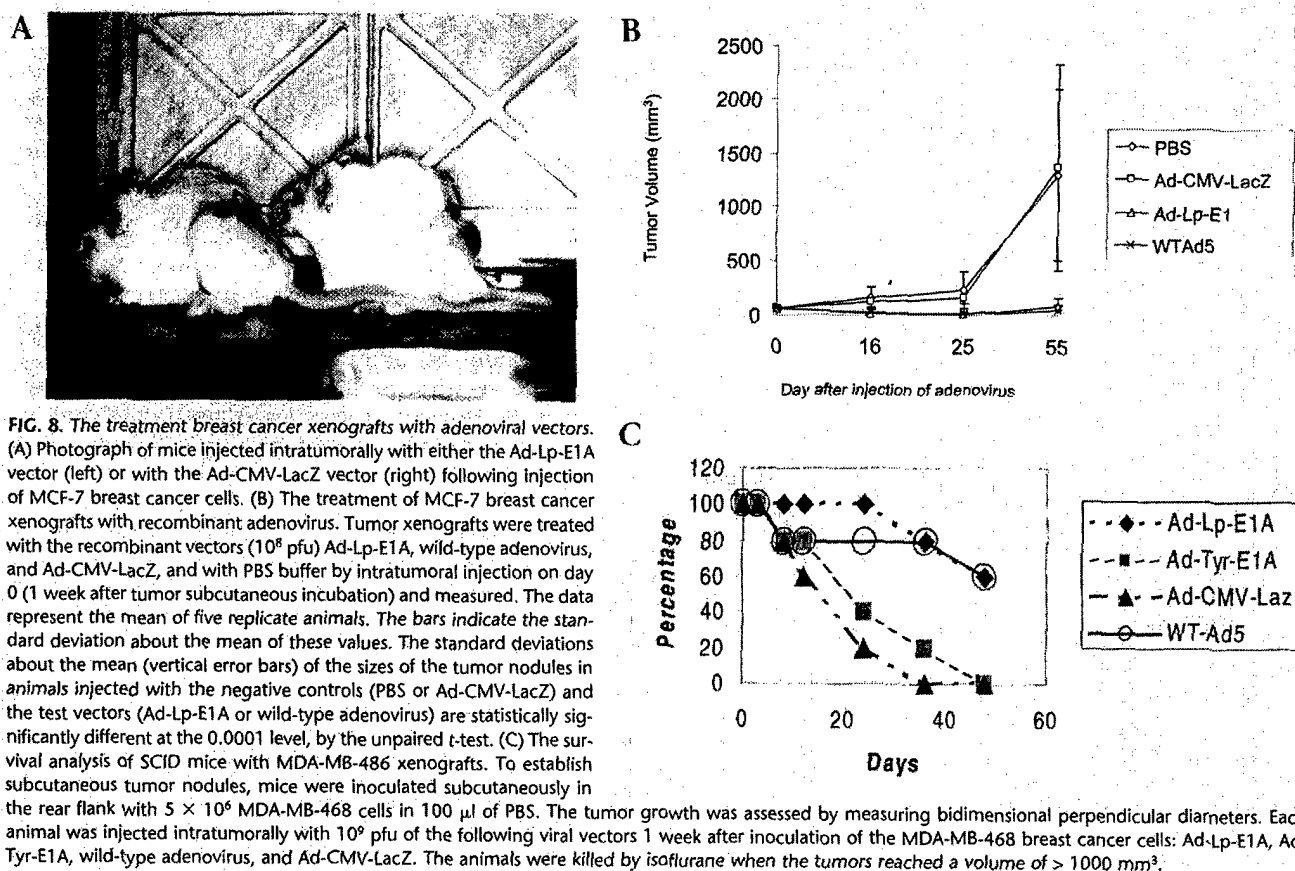


FIG. 7. Cytotoxicity of the adenoviral vectors to the melanoma TF-2 cells 9 days after infection. Monolayers of the human melanoma cell line TF-2 were exposed to wild-type virus (top left), the Ad-CMV-E1A vector (bottom left), the Ad-Lp-E1A vector (top right), and the Ad-Tyr-E1A vector (bottom right).

cell lines were injected subcutaneously into SCID mice, allowed to grow into subcutaneous tumor nodules of at least 50 mm³ in size, and injected three times with 1×10^8 pfu of the wild-type adenovirus (Fig. 8B), the Ad-Lp-E1A vector (Fig. 8B), or the Ad-CMV-LacZ vector (Fig. 8B), once every 2 days. Injection with the Ad-Lp-E1A vector was associated with inhibition of MCF-7 tumor cell growth (Figs. 8A and 8B). The standard deviations about the mean are defined for each data point by the vertical error bars. In Fig. 8B, the standard deviation about the mean of the sizes of the tumor nodules in animals injected intratumorally with the wild-type adenovirus or the Ad-Lp-E1A vector do not overlap with the standard deviation about the mean of the tumor sizes in animals injected intratumorally with PBS or with the Ad-CMV-LacZ vector. The differences between the standard deviation about the mean of the tumor nodule size following intratumoral injection with the Ad-Lp-E1A vector and the standard deviation about the mean of the tumor nodule size following intratumoral injection with the Ad-CMV-LacZ vector or PBS are statistically significantly different at the $P < 0.0001$ level, using the unpaired *t*-test. If one tests whether the means of the sizes of the tumor nodules injected with the Ad-Lp-E1A vector are different from the means of the sizes of the tumor nodules injected with the Ad-CMV-LacZ vector or PBS using the unpaired *t*-test, the two-tailed *P* value is 0.0152 or 0.0262, respectively, which is considered significant. These results indicate that injection of the Ad-Lp-E1A vector can suppress the growth of the MCF-7 cell line *in vivo*. A similar result was observed in MDA-MB-468 human breast cancer cell line xenografts (Fig. 8C). However, all injected cell lines eventually regrew in this model.

In a similar manner, the human melanoma TF-2 cell line was injected subcutaneously and allowed to grow into a tumor nodule in SCID mice to test the lytic effect of the Ad-Tyr-E1A vector on human melanoma cells *in vivo*. Then, these nodules were injected intratumorally once with 1×10^8 total particles of the vectors Ad-Tyr-E1A (Fig. 9), wild-type adenovirus (Fig. 9), and Ad-CMV-LacZ (Fig. 9), and with PBS (Fig. 9). The injection of the wild-type adenovirus into the TF-2 tumor nodules, or the injection of the Ad-Tyr-E1A vector into the tumor nodules, resulted in a reduction in the size of the melanoma tumor nodule, whereas no reduction in tumor growth occurred with injection of either PBS or the Ad-CMV-LacZ vector (Fig. 9). The standard deviations about the mean of the sizes of the tumor nodules injected intratumorally with the wild-type adenovirus or the Ad-Tyr-E1A vector were less than and did not overlap with the standard deviation about the mean of the tumor nodule sizes in animals injected intratumorally with PBS or with the Ad-CMV-LacZ vector. Although the TF-2 tumor nodules injected with the Ad-Tyr-E1A vector appeared to be growing more slowly than the TF-2 nodules injected with PBS or with the Ad-CMV-LacZ vector, the differences in the standard deviations of the means of the tumor sizes injected with Ad-Tyr-E1A versus PBS or Ad-CMV-LacZ were not statistically significantly different (*P* values 0.1178 or 0.1599, respectively), as determined by the unpaired *t*-test. This lack of statistical significance may reflect the wide range of replicate values in the PBS- and Ad-CMV-LacZ-injected tumor nodules. Longer follow-up showed that the wild-type adenovirus- or Ad-Tyr-E1A-vector injected TF-2 human melanoma cell line eventually regrew.



DISCUSSION

The goal of this work was to create adenoviral vectors that displayed tumor-specific replication competency and that were directly cytotoxic to tumor cells. In these replication-competent adenoviral vectors, we kept E1A and E1B intact because deletion of the E1B gene or the reduction of the expression of both the E1A and E1B genes through the use of an inducible promoter will decrease remarkably the

replication of these vectors [16]. In the vectors described in this paper, the E1A promoter region was deleted, but the enhancer that overlaps the 5' end of the viral packaging signal remains. This remaining viral element may enhance the L-plastin promoter, because the L-plastin promoter in the vectors we have developed appears to be stronger in the adenoviral vector than in plasmid expression vectors (data not shown). The L-plastin promoter is a tissue-specific but weak promoter.

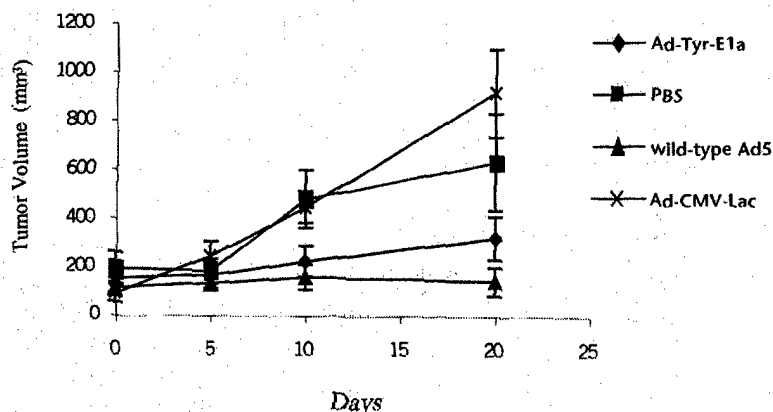


FIG. 9. The treatment of TF-2 melanoma tumor xenografts with recombinant adenoviral vectors. The tumor xenografts were injected intratumorally with 10^8 pfu of Ad-Tyr-E1A, wild-type adenovirus, or Ad-CMV-LacZ, or by PBS buffer, by intratumoral injection on day 0 (1 week after subcutaneous tumor cell inoculation) and measured weekly. The data represent the mean of five replicate animals. The bars represent the standard deviation about the mean. The standard deviations about the mean of the tumor sizes in animals injected with either PBS or Ad-CMV-LacZ were not found to be statistically significantly different with the standard deviation about the mean of the sizes of tumor nodules in animals injected with the Ad-Tyr-E1A vector ($P = 0.1178$ or 0.1599) by the unpaired t-test.

We chose to compare the cytotoxic action of replication-competent vectors, which contained the L-plastin promoter 5' to the E1A gene (Ad-Lp-E1A), with another vector that contained the tyrosinase promoter and tissue-specific enhancer 5' to the E1A gene (Ad-Tyr-E1A), with a vector in which the CMV promoter drove the E1A gene (Ad-CMV-E1A), and with the wild-type adenovirus. These experiments extended previous work in our laboratory with replication-incompetent adenoviral vectors, which showed that a truncated form of the L-plastin promoter was active in established neoplastic cell lines derived from estrogen-dependent tissues, even after being embedded in the adenoviral backbone [7,15].

We showed first that the Lp-E1A transcription unit of the Ad-Lp-E1A vector was expressed in breast cancer cell lines but not in explant cultures of normal human mammary epithelial cells. We showed as well that the tyrosinase-E1A transcription unit was not active in breast cancer cell lines. We then showed that the Ad-Lp-E1A was cytotoxic *in vitro* to the MCF-7 and the MDA-MB-468 established human breast cancer cell lines, destroying the entire monolayer at an MOI of 10, but was not toxic to normal human mammary epithelial cells at the same MOI. The Ad-Lp-E1A also caused lifting up and rounding up of all of the cells in explant cultures of ovarian cancer cells obtained from surgical specimens when the vector was added at an MOI of 10. This pattern of toxicity (Figs. 3–5), which mirrored the expression of the E1A transcription unit when placed under control of the L-plastin promoter (Fig. 2A), suggests that the L-plastin promoter is specifically active in cancer cells but not in normal cells, and that the Ad-Lp-E1A vector is selectively toxic to cancer cells but not to normal cells.

The Ad-Tyr-E1A vector was directly cytotoxic *in vitro* predominantly to tumor cell lines that corresponded to the origin of the tyrosinase promoter (melanoma cells), whereas they were much less toxic to the breast and ovarian cancer cells. The Ad-Lp-E1A vector was much more toxic to breast and ovarian cancer cells than to melanoma cell lines. Thus, the L-plastin-driven E1A vector was not only much more toxic to cancer cells than to noncancer cells of the same epigenotype, but this vector also was much more toxic to breast and ovarian cancer than to other types of nonepithelial cancer.

We were also able to show that this tumor-specific cytolytic effect extended into the *in vivo* setting by injecting the vectors into the subcutaneous nodules of either breast cancer cells or melanoma cells in a SCID mouse human tumor xenograft. These experiments showed that the Ad-Lp-E1A vector could transiently suppress the growth of the MCF-7 or MDA-MB-468 breast cancer cell lines and could suppress those cells even after they had grown into an established tumor nodule. In addition, the Ad-Tyr-E1A vector transiently suppressed the *in vivo* growth of the TF-2 melanoma cell line in the SCID mouse model. Because the injection of the Ad-CMV-LacZ

control replication-incompetent adenoviral vector into the subcutaneous tumor nodules in the SCID mice did not suppress the growth of the tumor nodules, the suppressive effect of the Ad-Lp-E1A and the Ad-Tyr-E1A vectors was most likely dependent on the replication competency of these two vectors, rather than to a consequence of an immunological reaction to the vector. In addition, the lytic effect of the Ad-Lp-E1A vector on cell lines *in vitro* was equivalent to the effect of the Ad-CMV-E1A on the tumor nodules in the SCID mice.

Thus, these data suggest that the Ad-Lp-E1A vector may ultimately be of value for the development of therapeutic vectors for the treatment of solid tumor malignancies. However, the suppressive effect of the vectors was not durable. It will be important to add a therapeutic transcription unit to these vectors so that their toxic action on tumor cells is more robust. A second change would be to engineer the vector such that it would bind only to tumor cells and not to normal cells. The effects of these changes are currently under study in our laboratory.

MATERIALS AND METHODS

Cells and cell culture. The following cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA): the MCF-7 and MDA-MB-468 human breast cancer cell lines and the 293 human embryonic kidney (HEK) cell line. The Ovar-5 human ovarian cancer cell line was obtained from Thomas C. Hamilton of the Fox Chase Cancer Center (Philadelphia, PA). The TF-2 and Yusac-2 human melanoma cell lines were obtained from Ruth Halaban at Yale University (New Haven, CT). Explants of normal human mammary epithelial cells were obtained from Clonetics (Waldersville, MD). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Structure of the L-plastin and tyrosinase promoter E1A replication-competent adenoviral vectors. A DNA fragment that contains the E1 gene was generated from wild-type adenovirus 5 using the following PCR primers: 5'-ACGCGTCGACGCGAGTAGAGTTTCTCTCCG-3' and 5'-AGCTTGTTTAACTCGAGGACAGGCCTCTCAAG-3' (GeneAmp XL PCR kit, Perkin Elmer). The 5200-bp PCR fragment was digested with *Sall* and *PmeI* restriction enzymes and then ligated into the pShuttle plasmid [14], which had been cut with *Sall* and *PmeI* to produce a new shuttle plasmid (pADshuttle) which contains a complete E1 gene but a deleted E1A promoter. The promoter fragments were inserted between the *NotI* and *Sall* sites of the pADshuttle (Fig. 1). The L-plastin *Scal* promoter was inserted into pBluescript Sk+ after it was excised from AdLpLaz [7]. The L-plastin *Scal* promoter fragment was then cut from the pBluescript Sk+ with *NotI* and *XhoI*. It was then ligated into the pAD shuttle plasmid. The human tyrosinase promoter-enhancer was synthesized by PCR from human Yusac-2 genome with three pairs of primers: HTP1, 5'-CCGGAATTCATTTCAACCATAAGAATTAA-3', and HTP2, 5'-ACGCGTCGACGGAACCTGGCTAATTGGAGTC-3'; TE1, 5'-ATTTCGCGCCGCAATTCGTCTTCGAGAACAT-3', and TE2, 5'-CGCGGATCCATGGAAATGCTGCCTCTG-3'; HEN1, 5'-CGCGGATCCAAATTCITCGAGAACAT-3', and HEN2, 5'-CCGGAATTCATGGAAATGCTGCCTCTG-3'. The HTP fragment was cut with *Sall* and *EcoRI*. The HEN fragment was cut with *EcoRI*. The vectors generated were sequenced. After sequencing, this *NotI* and *Sall* fragment was inserted into the pAD shuttle vector. We also synthesized the CMV promoter with PCR and inserted it into the pAD shuttle plasmid. The vector has the same regulatory elements left in the E1A and E1B region, including the vector-packaging signal, as reported by Rodriguez *et al.* [4].

The replication-competent adenoviral vectors under the control of the λ -plastin, tyrosinase, and CMV promoters were prepared by standard homologous recombination techniques using the pAD shuttle plasmids with AdEasy-1 (provided by Tong-Chuan He and Bert Vogelstein [14] of the Howard Hughes Medical Institute, Johns Hopkins Oncology Center) in Bjs138. After cutting with *PacI*, the adenovirus DNA plasmids, which contain different promoters, were transfected into HEK 293 cells. Each recombinant adenoviral vector was isolated from a single plaque and expanded in HEK 293 cells. Viral DNA was treated and analyzed by PCR to confirm the structure of the E1A promoters in the virus. The λ -plastin promoter was sequenced to confirm its structure.

The vector for experimentation was prepared by infecting 30 15-cm tissue culture plates of HEK 293 cells and by harvesting the detached cells after 48 hours. The viral particles remained associated with the cells. Cells were collected by centrifugation at 400g for 5 minutes at 4°C. The cells were resuspended in 10 mL of cold PBS (free of Ca^{2+} and Mg^{2+}), and were lysed with three cycles of freezing and thawing. The cells were collected by centrifugation at 1500g for 10 minutes at 4°C. The supernatant was placed on a gradient prepared with equal parts of cesium chloride in phosphate-buffered saline (PBS; 1.45 g/mL and 1.20 g/mL), and then centrifuged for 3 hours at 15,000g at 12°C. The virus band was removed, rebanded in a preformed cesium chloride gradient by ultracentrifugation for 18 hours, and dialyzed against cold PBS, pH 7.4, containing 10 mM MgCl_2 and 10% glycerol. Titers of purified adenoviral vectors were determined by spectrophotometry and by plaque assays.

Western blot analysis of adenovirus E1A protein expression. For detection of the E1A protein, 2×10^5 cells in six-well culture plates were infected with Ad-Lp-E1A and Ad-CMV-E1A at an MOI of 10. Cells were collected and lysed in cell lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 10 mM NaF, 10 ng/mL aprotinin, 10 ng/mL leupeptin, 1 mM DTT, and 1 mM sodium vanadate) 24 hours after viral infection, incubated for 60 minutes on ice, and centrifuged at 1500g for 10 minutes at 4°C. The supernatants were transferred to Eppendorf tubes and were kept at 100°C for 5 minutes. We added 10 μg of protein to each lane of the SDS PAGE gel. Protein was analyzed by immunoblotting with monoclonal antibody against the wild-type Ad5 E1A protein. Reactivity was visualized by enhanced chemiluminescence (Amersham Life Sciences, Inc., Arlington Heights, IL).

In vitro viral replication assay. Monolayer cell cultures in six-well dishes (1×10^5 cells/well) were infected with the Ad-Lp-E1A, Ad-Tyr-E1A, and the adenoviral vector with the CMV promoter driving the E1A gene (Ad-CMV-E1A), at an MOI of 10 pfu/cell. The virus was removed 2 hours after inoculation. The cells were then washed twice with PBS and incubated at 37°C for 72 hours. Lysates were prepared with three cycles of freezing and thawing. Serial dilutions of the lysates were titered on HEK 293 cells.

Cytopathic effect assays. Cells were passaged 24 hours before infection with adenoviral vectors at the indicated MOI. Photomicrographs were taken on days 3, 5, and 9 after infection.

In vivo gene transfer to human breast cancer xenografts and human melanoma xenograft. Subcutaneous tumor nodules were established by subcutaneous injection of $5\text{--}10 \times 10^6$ MDA-MB-468, MCF-7 breast cancer cells or TF-2 melanoma cells suspended in 0.1 mL of PBS into the flanks of female SCID mice aged 5–6 weeks. Tumor nodules were allowed to grow subcutaneously to approximately 6–7 mm in diameter. For intratumoral

injection of the viral vectors, 50 μL of viral particles (10^8 pfu) suspended in PBS were injected using a 25-gauge needle. Tumor size was measured at the indicated times after injection in their longest dimension and the dimension at 90° to that measurement. Tumor volumes were calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. Tumor volumes were normalized to 100% on day 0 (V/V_0). Results are expressed as the fractional tumor volume (mean \pm SD) at each time point compared with that on the day of injection.

ACKNOWLEDGMENTS

We recognize support to A.D. from the United States Army (DAMD 17-99-9457), The Audrey Demas Melanoma Research Fund, The Anthony Dewitt Frost Memorial Fund for Melanoma Research, The Greenwich Breast Cancer Collective, The Hull Development Fund, The Breast Cancer Research Foundation, The Susan Komen Foundation, and the George and Barbara Bush Leukemia Research Fund.

RECEIVED FOR PUBLICATION APRIL 12, 2001; ACCEPTED JULY 22, 2002.

REFERENCES

- Haj-Ahmad, Y., and Graham, F. (1986). Characterization of an adenovirus type 5 mutant carrying embedded inverted terminal repeats. *Virology* 153: 22–34.
- Bett, A., Haddara, W., Prevec, L., and Graham, F. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* 91: 8802–8806.
- Pang, S. (2000). Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. *Cancer Gene Ther.* 7: 991–996.
- Rodriguez, R., et al. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* 57: 2559–2563.
- Miyatake, S. I., et al. (1999). Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus in vivo. *Gene Ther.* 6: 564–572.
- Heise, C., and Kirn, D. H. (2000). Replication-selective adenoviruses as oncolytic agents. *J. Clin. Invest.* 105: 847–851.
- Chung, I., Schwartz, P. E., Crystal, R. G., Leavitt, J., and Deisseroth, A. B. (1999). Use of λ -plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Ther.* 2: 99–106.
- Lin, C. S., et al. (1997). The murine λ -plastin gene promoter: identification and comparison with the human λ -plastin gene promoter. *DNA Cell Biol.* 16: 9–16.
- Lin, C. S., et al. (1993). Characterization of the human λ -plastin gene promoter in normal and neoplastic cells. *J. Biol. Chem.* 268: 2793–2801.
- Lin, C. S., et al. (1993). Human plastin genes. Comparative gene structure, chromosome location, and differential expression in normal and neoplastic cells. *J. Biol. Chem.* 268: 2781–2792.
- Siders, W. M., et al. (1998). Melanoma-specific cytotoxicity induced by a tyrosinase promoter-enhancer/herpes simplex virus thymidine kinase adenovirus. *Cancer Gene Ther.* 5: 281–291.
- Siders, W. M., et al. (1998). T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. *J. Immunol.* 160: 5465–5474.
- Siders, W. M., et al. (1996). Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res.* 56: 5638–5646.
- He, T. C., et al. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95: 2509–2514.
- Peng, X. Y., Rutherford, T., Fujii, T., Schwartz, P., and Deisseroth, A. (2001). The use of the λ -plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res.* 61: 4405–4413.
- Ohashi, M., et al. (2001). Target gene therapy for α -fetoprotein-producing hepatocellular carcinoma by E1B55k-attenuated adenovirus. *Biochem. Biophys. Res. Commun.* 282: 529–535.

Molecular chemotherapy

ALBERT DEISSEROTH, M.D.^{1,2}, TAKUMA FUJII, M.D.^{1,2,6}, XUEYUAN PENG, M.D.^{1,2}, LIXIN ZHANG, M.D.^{1,2}, DAVID AUSTIN, PH.D.^{5,2}, THOMAS RUTHERFORD, M.D.^{2,3}, JANET BRANDSMA, PH.D.^{2,4}, PETER E. SCHWARTZ, M.D.^{2,3}

Medical Oncology Section, Department of Internal Medicine¹, Genetic Therapy Program², Yale Cancer Center, Gynecologic Oncology Section, Department of Obstetrics and Gynecology³, Department of Comparative Medicine⁴, Department of Chemistry⁵, Yale University School of Medicine, Department of Obstetrics and Gynecology⁶, Keio University School of Medicine

ABSTRACT For the past 50 years, during which chemotherapy has been administered for the treatment of cancer, the paradigm driving the selection of chemotherapeutic agents was their capability to damage the DNA of dividing cells. In addition, chemotherapeutic agents were studied for the selectivity of their toxic effect to the cancer cell versus the normal cell. Because many of the normal cells in the body shared the phenotype of cellular division with the tumor cells, the drugs developed for the treatment of cancer displayed only relative selectivity for the cancer cell. The advent of structural biology and computational chemistry to drug development, and the explosion of information about the molecular and genetic changes acquired in the cancer cell, have now produced the opportunity to design drugs for cancer treatment which specifically block the effects of the signals within cancer cells which drive the evolution of the disease process to unregulated cell growth. The implementation of this new mode of drug design to the field of cancer chemotherapy is the subject of this chapter.

Key words molecular chemotherapy, gynecologic malignancies

INTRODUCTION One of the obstacles to the success of chemotherapy for cancer treatment is that resistance may evolve very rapidly following exposure to chemotherapy, or is present at the start of chemotherapy. Thus, remissions are fol-

lowed by recurrences and the phenotype of the recurrent disease is much more resistant than that of the original tumor population.

This pattern of recurrence is often assumed to be due to the genetic instability of tumor cell. The imposition of negative selection on any population of genetically unstable cells leads to low frequency clones of tumor cells which may have acquired higher levels of resistance.

NORMAL TISSUES ASSOCIATED WITH TUMORS ARE NOW TARGETS FOR MOLECULAR CANCER TREATMENT The acquisition of additional somatic mutations due to the error prone nature of DNA replication, the increased proliferative activity of tumor populations, and the diminished repair capacity of mutant populations of neoplastic cells, leads to increasing resistance in tumor cells to the effects of chemotherapy. This has led many therapists to consider changing the target of therapy from the tumor cells themselves to alternative tumor associated populations of normal cells upon which the continued proliferation or survival of the tumor population depends. One such target is the vasculature associated with tumor tissue. Another normal target which can affect the natural history of a tumor population is the systemic immune response.

DEVELOPMENT OF MOLECULAR THERAPY INVOLVING TUMOR VESSELS *Folkman* (1) has emphasized the important role that the elaboration of the tumor associated vasculature plays in the growth of solid tumors. It is clear that for tumor cells to grow above a minimal size, which is determined by the diffusion limits of nutrients and oxygen, the tumor must stimulate the development of vasculature as the tumor grows. The normal endothelial cells start to extend into the growing tumor mass, perhaps preceded by the influx of pericytes. *Folkman* (1) has focused on the development of agents which are known to suppress the growth of endothelial cells, the so called tumor neovasculature. Although this "anti-angiogenic" therapy has displayed activity in animal models, it may result only in stabilization of the size of tumor nodules. This is due to the fact that the anti-angiogenic therapy does not affect the existing vasculature, but only the development of additional vasculature which then allows the tumor to grow.

This recognition has led to the study of the tumor vasculature, both in the growing zone of angiogenesis and in the stable non-proliferating zone of the established tumor mass. Interestingly, both the established vasculature of tumor tissue, as well as the growing zone of tumor neovasculature, display proteins on the endothelial surface that do not appear on the surface of endothelial cells which are part of vessels of normal tissue. *Schmitzer and co-workers* (2) have reported the appearance of a number of novel proteins on the surface of tumor

Address correspondence to:

Albert Deisseroth, M.D.
Yale University School of Medicine
333 Cedar Street, New Haven, CT 06520-8032, USA
Phone (1 203) 737 5608 Fax (1 203) 737 56989
E-mail adeisseroth@skcc.org

cell vasculature. Other workers have developed data suggesting that the established vessels of tumor tissues display tissue factor, a transmembrane protein not usually exposed to the luminal surface of normal vessels in the absence of vessel injury or inflammation (3-4). When tissue factor is exposed in the setting of vascular injury or inflammation to the luminal surface of vessels, it binds to factor VII, which is a factor of intravascular coagulation. Upon binding of factor VII to tissue factor, factor VII is activated thereby triggering the extrinsic pathway of coagulation which results in the conversion of fibrinogen into a fibrin clot.

The exposure of the blood stream to tissue factor and the consequent binding and activation of factor VII also has an effect on the activation of platelets as well. Since tissue factor does not appear on the endothelial cells of vessels associated with normal tissue, the presence of tissue factor on the luminal surface of the endothelium of tumor associated vessels has provided a target to which to selectively deliver anti-cancer therapeutic reagents.

Another way of identifying molecular targets which can be used to deliver therapeutics to tumor vasculature is in vivo phage display. *Pasqualini and Ruoslahti* (5) have injected into tumor bearing mice a library of bacteriophage, which display random peptides on the surface of the coat proteins of the bacteriophage. Injection of these bacteriophage showed a four-fold difference in the binding of the various bacteriophage clones to vessels of different tissues. Also, peptides which bind to known proteins present on tissues (6) were isolated (e.g. the RGD peptide which is known to bind to the alpha V beta 3 integrin receptor present on the endothelial cells of some tissues). These workers are attempting to determine if this pattern of binding of random peptides to the endothelial cells of different types of tissues can be used to target therapeutics to tumor associated vasculature.

Other tumor vascular targeting approaches which may reach the clinic include adenoviral vectors which are engineered to bind to tumor neovasculature, or which carry transcription units encoding proteins directed to the tumor vasculature, or chemicals which can target directly the tumor vasculature or angiogenesis receptors.

At the beginning of the field of anti-angiogenesis therapy, studies focused on natural products and low molecular weight chemicals which target tumor vessels or angiogenesis receptors. *Folkman* (1) has been responsible for launching therapeutic trials of number anti-angiogenic agents including endostatin and angiostatin which are derived from normal proteins in human beings. The animal experiments designed to analyze the efficacy of these proteins involved the infusion of the recombinant proteins on a daily basis to patients with a wide

spectrum of malignancies. The data so far from these clinical phase I trials suggest that the incidence of partial responses will be low, and that the most that one can hope for may be stabilization of disease.

These results have prompted investigators to combine anti-angiogenic proteins with chemotherapy or radiation therapy. Such trials are currently underway. The second direction is to engineer an angiostatin or endostatin expression unit in the backbone of the adenoviral vector (7). These efforts are currently at the preclinical and animal model stage. Presumably the advantage of using vectors for the delivery of the recombinant endostatin or angiostatin proteins is that instead of a daily infusion, which results in wide fluctuations of the intravascular levels of these proteins, the use of a vector produces continuous levels of the therapeutic protein. The limitation of this approach may be the absolute level of that protein can be maintained and the time over which one can administer these vectors. The adenovirus stimulates a strong immune response. There may therefore be a limit to the number of injections one can administer before the host immune response may block the effect of the vectors.

A final vector approach to tumor neovasculature is to engineer adenoviral vectors carrying transcription units encoding chimeric immunoconjugate molecules which are composed of an aminoterminal targeting domain and a carboxylterminal functional domain. In one of these, *Hu et al.* (8-9) have introduced into a replication incompetent adenoviral vector a transcription unit which encodes the aminoterminal domain of a modified factor VII carrying a loss of function mutation, which can bind to the tissue factor on the surface of the tumor associated vasculature, linked to the carboxylterminal end of the IgG Fc fragment of an immunoglobulin molecule. The strategy is to inject the adenoviral vector encoding this immunoconjugate molecule into a subcutaneous tumor nodule. The immunoconjugate transcription unit is engineered such that the fusion immunoconjugate factor VII/Ig G Fc protein is secreted from the vector infected tumor cells into the systemic circulation where it is free to bind to vessels associated with tumor cells all over the body. Preclinical animal studies of this approach so far published (8-9) show that metastatic deposits far from the nodules undergo regression. This approach is undergoing extensive preclinical testing at this time.

A non-vector approach is to design low molecular weight chemicals which can target the endothelial surfaces of vessels associated with tumor cells. These drugs are based on the analysis of the peptides which are found to bind to tumor associated vessels, or the computational analysis of receptors which participate angiogenesis. These directions will be pursued in the next two to three years.

DEVELOPMENT OF MOLECULAR THERAPY INVOLVING THE IMMUNE RESPONSE The other normal tissue which can affect the natural history of tumor cells is the immune response. Many workers are exploring ways of producing novel drugs, vectors and recombinant proteins which can be used to target the immune response against the tumor cells.

The explosion of information about antigen presentation in normal tissues and tumor tissues suggests that there are many abnormalities in the presentation of tumor antigens. Decreases in the level of surface cytoadhesion molecules necessary for presentation of antigens are characteristic of tumor cells as compared to normal cells. Another major problem is that the tumor antigens are transmitted only very inefficiently to the intracellular environment of professional antigen presentation cells, the dendritic cells. The start of the immune response is the presentation of the target antigen by specialized cells which display antigenic peptides in a context which can be recognized by clones of T cells competent to respond to these antigens (10).

This system works very well to limit and control viral infections. Following the infection of respiratory mucosal cells by viruses, the dendritic cells, which are present in large numbers in these surfaces, are infected. The replication of the vectors then results in the elaboration of high levels of viral proteins within the dendritic cells. This results in the appearance of high levels of viral specific peptide antigens bound to histocompatibility presentation molecules associated with beta 2 microglobulin. This generates a systemic immune response against the virus and virally infected cells.

Although dying tumor cells can release tumor associated proteins which can be taken up by antigen presenting cells, this process is very inefficient and therefore the levels of tumor associated proteins within the antigen presenting cells is very low. Thus, there is no mechanism through which tumor associated proteins can reach levels within the antigen presenting cells which are in excess of the endogenous proteins of the antigen presenting cell. This results in levels of tumor-associated peptides bound to antigen presentation molecules which are low in comparison to non-tumor associated proteins on the surface of antigen presenting cells. The only circumstance under which tumor cell antigens can be presented at very high levels on antigen presenting cells is in the neoplastic diseases in which the dendritic cells can differentiate from the neoplastic population itself (11). In the case of lymphomas and leukemias, in which this can take place, there is good evidence that autologous dendritic cells, and the infusion of T cells exposed to such dendritic cells, can induce regressions of established tumors, or prevent the engraftment of tumor cells in animal models (12).

For most tumors however, the only mechanism which can deliver exogenous tumor associated proteins to the intracellular compartment of the dendritic cell such that they can appear on the surface of the dendritic cell in a way that triggers a T cell cellular immune response to the tumor antigens, is the process which is called cross priming (13).

Purified or recombinant proteins, peptides and gangliosides have been used in an attempt to activate the cellular immune response. Peptides administered alone or with adjuvants, or peptides linked to molecules which can target sites on antigen presenting cells, or to subcellular locations within dendritic cells, are the subject of intense preclinical and clinical investigation at this time (14-15).

Many papers report animal model data based on the use of adenoviral vectors carrying transcription units which code for tumor associated antigens. These adenoviral vectors are used to infect the dendritic cells. These infected dendritic cells are infused into the intravenous space, injected into the lymph nodes, injected into tumor cells, or injected into the subcutaneous or intradermal space. Dendritic cells have also been loaded by fusing the dendritic cells with tumor cells to introduce the tumor antigens into the antigen presenting cell (16). Finally, incubation of the antigen presenting cell with recombinant peptides that bind to the HLA peptide binding pocket has been used to load dendritic cells with tumor associated antigens. These molecular targeting approaches are currently under study in preclinical and clinical trials.

APPROACHES FOR THE DESIGN OF MOLECULAR TARGETED DRUG THERAPY Computational and combinatorial chemistry can be used for the development of molecular approaches, which target the tumor cell. The approach differs when structural data about the target protein is available, and in those cases in which there is no data about the structure of the target protein.

DESIGN OF MOLECULAR THERAPEUTICS STARTING FROM PROTEIN STRUCTURE DATA Ample data now exists that supports the contention that oncoprotein structures can be used to design inhibitors of enzymes or proteins associated with human disease. The first example is the use of X-ray crystal structure to design inhibitors of proteases which are necessary for the continued replication of the human immunodeficiency virus. Recently, *Thiesing et al.* (17) has brought forward a drug, STI 571, which blocks the binding of ATP to the ATP binding site of the enzyme pocket of the p210bcrabl kinase for the treatment of chronic myelogenous leukaemia (CML).

Models for the development of drugs which can bind to targets which are unique to tumor cells are under development for many different types of targets in tumor cells: for growth

factor receptors, metastasis receptors, purine pockets in membrane G proteins and oncoprotein tyrosine specific protein kinases. Pockets or receptors are the best targets for this type of drug design rather than the protein contact points which often do not have single points at which the stability of a complex can be affected.

In addition to the use of computational analysis of purine pockets to design chemicals which can inhibit the function of oncoproteins, chemical combinatorial libraries can be generated which are biased on the basis of the structure of lead compounds derived from structural analysis of the target oncoproteins or their co-factors. Recombinant fragments of the target protein are then used to screen the chemical combinatorial libraries for compounds which exhibit high affinity or inhibitory activity for the target proteins. In addition, molecular scaffolds attached to the lead compounds, which project chemical functionalities in multiple directions, can be subjected to combinatorial structural diversification (18). The library is screened with recombinant clones of the target protein for compounds binding the target at multiple independent protein amino acid motifs. This may produce compounds which can inhibit target proteins by binding at multiple sites. This theoretically can produce inhibitors which are very selective and are high affinity binders.

DESIGN OF MOLECULAR THERAPEUTICS WHICH DO NOT DEPEND ON AVAILABILITY OF PROTEIN STRUCTURE The algorithm outlined above for the development of drugs based on the analysis of protein structure is different when there is no X-ray crystallographic data or nuclear magnetic resonance imaging based on structural data. The design of cancer treatment drugs without structural data is based on two approaches.

1. Use of computational chemistry to provide information about what kinds of chemical functionalities will bind to catalytic sites of the oncoprotein targets. Another example is the development of mimics for growth factors which are inhibitory for growth factor receptors. Once chemical mimics are designed for particular targets, then cell line experiments, followed by animal model experiments are carried out to test the safety and efficacy of the candidate compounds.

2. The other basic approach uses recombinant fragments of the target protein to screen phage display random peptide libraries for peptide which bind to the target protein. The next step is to identify the common amino acid motifs in the peptides which confer binding specificity to the oncoprotein targets. Site directed mutagenesis is then used to identify which amino acid moieties necessary for binding. Computational chemistry is then used to design of low molecular weight chemical functionalities which can block the function of the on-

coprotein upon which the transformed phenotype of the cancer cell depends.

At the end, combinatorial libraries can be generated which are based on mimics of peptides which bind to the target proteins. The combinatorial libraries contain the bias of the mimics as the binding domain, but also contain 4 or 5 other chemical functionalities, each projecting from a different position on the scaffold, which can be independently varied structurally. This creates inhibitors which bind to 4 or 5 independent domains at the target site. The chemical inhibitors thereby produced can often bind with very high affinity and very selectively.

Using this approach for drug design, low molecular weight chemicals are being developed for the following targets: growth factor receptors, metastasis receptors, replication complexes of DNA tumor viruses, and DNA tumor viruses transforming proteins. In addition, normal protein targets such as anti-angiogenesis inhibitors are being developed as well.

APPROACHES FOR THE DESIGN OF MOLECULAR THERAPEUTICS FOR GYNECOLOGICAL DISEASES

DESIGN OF MOLECULAR THERAPEUTICS FOR HPV ASSOCIATED NEOPLASMS Human papilloma virus (HPV) associated neoplasms represent the second leading cause of cancer death among women in the world (19). In developing nations, HPV associated neoplasms represent the leading cause of death among women between the ages of 25 and 35. In the United States, cervical cancer has been controlled for the majority of individuals who participate in yearly screening with the PAP smear. Chronic dysplasia associated with high-risk gene type of HPV infections which place women at a very high risk of developing cervical cancer. However, this transition from a dysplastic mucosa, in which the HPV DNA is replicating as an episome, and frankly invasive cancer, in which the HPV DNA is integrated into the host cell DNA, may require years.

Most people clear the HPV infection spontaneously. However, a small percentage of individuals fail to clear the infection possibly due to a defect in antigen presentation. These are the individuals in whom there is a high risk of developing cervical cancer. The PAP smear picks up these individuals. If the dysplasia is severe, surgical interventions are utilized to treat these individuals. In developing nations, there is no infrastructure for the screening of individuals who have cervical dysplasia or who are positive for chronic HPV infections.

Among immunodeficient individuals, HPV infections are much more extensive and may spread to the rectal and anal mucosa. Chronic infection in immunodeficient individuals places the individuals at risk of developing rectal cancer.

A PAP smear for the rectal mucosa has been developed for HPV infections of these individuals as well.

Two approaches are being used to attempt to prevent cervical cancer: vaccines which would make women and men all over the world resistant to infection by HPV, and drugs which are specifically targeted to suppress the replication of the HPV virus.

Computational chemistry was used to make predictions about the types of chemicals which might suppress the replication of the HPV through disruption of the replication initiation complex on the LCR of the viral DNA in the laboratory of David Austin. These data indicated that the indole nucleus might be useful as an inhibitor of replication of HPV, which depends on E2 and E1.

Fujii *et al.* (20), working in the Deisseroth laboratory, found that the indole nucleus appeared in random peptides which bind to HPV E2. This work suggested that the indole nucleus would be important as a basis on which to build low molecular weight chemical inhibitors of HPV replication. This data led to the funding of a multi-institutional National Cancer Institute trial designed to study the feasibility of using the indole nucleus to suppress chronic infections associated with HPV. At the same time, plans were developed to use combinatorial and computational chemistry to develop new inhibitors for the replication of HPV (18).

DEVELOPMENT OF MOLECULAR THERAPEUTICS BASED ON REPLICATION COMPETENT ADENOVIRAL VECTORS FOR OVARIAN CANCER Ovarian cancer is restricted to the peritoneal cavity in the majority of patients even at advanced stages of disease (21). However, 80% of patients are unresectable due to the dissemination of the ovarian cancer from the primary tumor to multiple sites within the peritoneal cavity as microscopic disease in multiple areas throughout the peritoneal cavity. These microscopic implants lead to relapse in ovarian cancer patients.

Intracavitary chemotherapy and abdominal radiation therapy have both failed to prevent this from happening in the majority of patients, because the ovarian cancer cells are more resistant to the effects of chemotherapy and radiation therapy than are the normal peritoneal lining cells. With the advent of adenoviral vectors and genetic therapy, there is an expectation that ovarian cancer could be treated with genetic or molecular therapy.

Our group has developed a series of adenoviral vectors designed to exhibit selective toxicity towards ovarian cancer without damaging the normal peritoneal lining cells of the peritoneal cavity. The start of this project was with the discovery made by Chung *et al.* (22) that the L-plastin promoter, when

placed in the backbone of the adenoviral vectors, retained its selectivity of expression on estrogen dependent tumor tissue, and was not actively expressed in the normal peritoneal lining cells (23). This promoter was then placed 5' to the E1A gene of the adenoviral vector, which the vector needs to be replication competent and directly lyse tumor cells (24). This produced a vector which was directly cytolytic to ovarian cancer cells, but which was not toxic to normal peritoneal lining cells (24). The next stage was to place a prodrug activation transcription unit in the conditionally replication competent L-plastin/E1A adenoviral vector. Finally, the next phase will be to engineer the fibrillar protein so that the adenoviral vector will bind to tumor cells but not to the normal peritoneal cells. These vectors are being studied in pre-clinical animal models.

SUMMARY OF MOLECULAR APPROACHES TO CANCER TREATMENT

It is clear that the advent of genetics, molecular biology and the ability to transfer structural information about oncoproteins into chemical functionalities, has forever changed the prospects for the development of a new generation of therapeutics which will exhibit greater degrees of selectivity and greater efficacy. Already, the vanguard of this new generation of therapeutics has reached the clinic. The early success with these agents has raised the hopes that these compounds will produce dividends for cancer patients all over the world. In addition, the increased selectivity of these compounds will reduce the suffering of patients and reduce the burden imposed on society due to toxicity.

ACKNOWLEDGEMENTS The authors recognize support from the Bush Leukemia Research Foundation, The Ensign Professorship of Medicine, the Hull Development Fund of the Yale Cancer Center, and the following grants from the US Army, The Breast Cancer Research Foundation, The Audrey Demas Fund, The Greenwich Breast Cancer Collective, and the American Cancer Society.

REFERENCES

1. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; 285:1182-1186.
2. Jacobson VS, Stolz DB, Schnitzer JE. Identification of endothelial cell-surface proteins as targets for diagnosis and treatment of disease. *Nature Medicine* 1996; 2:482-485.
3. Callender NS, Varki N, Rao LVM. Immunohistochemical identification of tissue factor in solid tumors. *Cancer* 1992; 70:1194-1201.
4. Nemerson Y. Tissue factor and hemostasis. *Blood* 1988; 71:1-8.
5. Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. *Nature* 1996; 380:364-366.
6. Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nature Biotechnol* 1998; 15:542-546.
7. Sauter BV, Martinet O, Zhang WJ, Mandeli J, Woo SL. Adenovirus-mediated gene transfer of endostatin in vivo results in high level of transgene expression and inhibition of tumor growth and metastases. *Proc Natl Acad Sci USA* 2000; 97:4802-4807.
8. Hu ZW, Sun Y, Garen A. Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. *Proc Natl Acad Sci USA* 1999; 96:8161-8166.

9. Hu AW, Garen A. Intratumoral injection of adenoviral vectors encoding tumor-targeted immunoconjugates for cancer immunotherapy. *Proc Natl Acad Sci USA* 2000; 97:9221-9225.
10. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392:245-252.
11. Choudhury A, Gajewski JL, Liang JC, Popalt U, Claxton DFR, Kliche KO, et al. Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood* 1997; 89:1133-1139.
12. Kolb HJ, Schattenberg A, Goldman JM, et al. Graft versus leukemia effects of donor lymphocyte transfusions in marrow grafted patients. *Blood* 1995f; 86:2041-2048.
13. Bevin MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 1976; 143:1283-1288.
14. Huang TH, Wu PY, Lee CN, Huang HI, Hsieh SL, Jung J, et al. Enhanced antitumor immunity by fusion of CTLA-4 to a self tumor antigen. *Blood* 2000; 96:3663-3670.
15. Lin KY, Guarniere FG, Stveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; 56:21-26.
16. Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nature Med* 1997; 3:558-561.
17. Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI 571, an Abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against Bcr-Abl-positive cells. *Blood* 2000; 96:3195-3199.
18. Deisseroth A, Psyrri A, Austin DJ. Molecular Therapy. In: *Principles and Practice of Oncology*. DeVita VT Jr, Hellman S, Rosenberg SA, eds. 6th edn. Lippincott-Raven, New York, 2000: 3180-3187.
19. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1990; 80:827-841.
20. Fujii T, Austin D, Deisseroth A. Peptide ligands for HPV 16 E2 isolated from a random peptide library. Submitted 2000.
21. McGuire WP, Hoskins WJ, Brady MF, et al. Cyclophosphamide and cisplatin compared with preclinical and cisplatin in patients with stage III and stage IV ovarian cancer. *N Eng J Med* 1996; 334:1-6.
22. Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, Deisseroth AB. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Therapy* 1999; 6:99-106.
23. Peng XY, Rutherford T, Wong H, Pizzorno G, Sapi E, Kaczinski B, et al. The use of the L-plastin promoter for adenoviral mediated tumor specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res* 2001; 61:4405-4443.
24. Zhang L, Peng XY, Pizzorno G, Deisseroth A. Tumor specific promoters confer tumor specific cytolytic action on adenoviral vectors in estrogen dependent tumors and melanoma. Submitted, 2000.

115. Barth A, Wanek LA, Morton DL. Prognostic factors in 1,521 melanoma patients with distant metastases. *J Am Coll Surg* 1995;181:193-201.
116. Keilholz U, Conradt C, Legha SS et al. Results of interleukin-2-based treatment in advanced melanoma: a case record-based analysis of 631 patients. *J Clin Oncol* 1998;16:2921-2929.
117. Eton O, Legha SS, Moon TE et al. Prognostic factors for survival of patients treated systemically for disseminated melanoma. *J Clin Oncol* 1998;16:1103-1111.
118. Brand CU, Ellwanger U, Stroebe W et al. Prolonged survival of 2 years or longer for patients with disseminated melanoma. *Cancer* 1997;79:2345-2353.
119. Bowen GM, Chang AE, Lowe L et al. Solitary melanoma confined to the dermal and/or subcutaneous tissue: evidence for revisiting the staging classification [in process citation]. *Arch Dermatol* 2000;136:1397-1399.
120. Deichmann M, Benner A, Bock M et al. S100-Beta, melanoma-inhibiting activity, and lactate dehydrogenase discriminate progressive from nonprogressive American Joint Committee on Cancer stage IV melanoma. *J Clin Oncol* 1999;17:1891-1896.
121. Franzke A, Probst-Keppler M, Buer J et al. Elevated pretreatment serum levels of soluble vascular cell adhesion molecule 1 and lactate dehydrogenase as predictors of survival in cutaneous metastatic malignant melanoma. *Br J Cancer* 1998;78:40-45.
122. Sirott MN BD, Wong GYC, Tao YT et al. Prognostic factors in patients with metastatic malignant melanoma. A multivariate analysis. *Cancer* 1993;72:3091-3098.
123. Agrawal S, Yao T-J, Coit DG. Surgery for melanoma metastatic to the gastrointestinal tract. *Annals of Surgical Oncology* 1999;6:336-344.
124. Bevilacqua RG, Coit DG, Rogatko A et al. Axillary dissection in melanoma: prognostic variables in node-positive patients. *Ann Surg* 1990;212:125-131.
125. Balch C. Surgical treatment of advanced melanoma. In: Balch C, Houghton AN, Sober AJ, Soong S-J, eds. *Cutaneous Melanoma*. St. Louis: Quality Medical Press, 1998:373-388.
126. Ollila DW, Hsueh EC, Stern SL et al. Metastasectomy for recurrent stage IV melanoma. *J Surg Oncol* 1999;71:209-213.
127. Wong JH, Skinner KA, Kim KA et al. The role of surgery in the treatment of nonregionally recurrent melanoma. *Surgery* 1993;113:389-394.
128. Eton O, Legha SS, Moon TE et al. Prognostic factors for survival of patients treated systemically for disseminated melanoma. *J Clin Oncol* 1998;16:1103-1111.
129. Bostick PJ, Morton DL, Turner RR et al. Prognostic significance of occult metastases detected by sentinel lymphadenectomy and reverse transcriptase-polymerase chain reaction in early-stage melanoma patients. *J Clin Oncol* 1999;17:3238-3244.
130. Curry BJ, Farrelly M, Hersey P. Evaluation of S-100 beta assays for the prediction of recurrence and prognosis in patients with AJCC stage I-III melanoma. *Melanoma Res* 1999;9:557-567.
131. Kelley MC, Ollila DW, Morton DL. Lymphatic mapping and sentinel lymphadenectomy for melanoma. *Semin Surg Oncol* 1998;14:283-290.
132. von Schoultz E, Hansson LO, Djureen E et al. Prognostic value of serum analyses of S-100 beta protein in malignant melanoma. *Melanoma Res* 1996;6:133-137.

Prevention of Cervical Cancer: Chemoprevention

Hakan Akbulut

Albert Deisseroth

Cervical cancer is the third most common cancer in women worldwide and is a major cause of overall mortality among young women in developing countries.¹ By the end of 2001, approximately 12,900 new cases of cervical cancer and 4400 deaths from this cancer are estimated to occur in the United States.² Since the introduction of regular gynecologic examinations and screening Papanicolaou (Pap) smear tests, the incidence and mortality of cervical cancer have decreased in developed countries,³ although for the last decade, the decline in incidence and mortality in those countries has begun to reverse.^{3,4} Meanwhile, the prognosis of cervical cancer patients is somewhat better than that for patients with most other solid tumors because of screening programs. Patients with early-stage disease have 5-year survival rates of approximately 80%,^{5,6} whereas this rate in patients with advanced disease does not exceed 10%. Currently, no efficient methods exist for treating the cancer in this group of patients.

The screening programs are useful not only for making an early diagnosis of cancer but also for identifying premalignant lesions that carry high potential for cervical cancer. Since the term *carcinoma in situ* (CIS) for premalignant lesions was first introduced almost 100 years ago, the spectrum of cervical mucosal abnormalities leading to invasive cervical cancer has been well defined. The

precursor lesions vary from mild to severe dysplasia and CIS. Richart's term, *cervical intraepithelial neoplasia* (CIN), refers to all lesions representing different stages of the same disease process for cervical cancer. The simplicity of the CIN terminology, classified as CIN1-CIN3 and representing the degree of dysplasia (from mild to severe) and CIS, has led to its wide use in clinical trials. The Bethesda System for Reporting Cervical/Vaginal Cytological Diagnoses classifies the lesions as *low-grade squamous intraepithelial lesions*, which are equivalent to CIN1, or *high-grade squamous intraepithelial lesions*, including the CIN2-CIN3 and CIS lesions.⁸ Abnormalities that do not fulfill the criteria for squamous intraepithelial lesions are defined as *atypical squamous cells of undetermined significance*. Although this new system has been used widely for the reporting of Pap test results, it is not used widely in clinical prevention trials.

Although previously reported studies on cervical dysplasia demonstrate a degree of selection bias, misclassification of cytopathologic findings, and neglect of some of the known important risk factors, they have shed light on the natural history of the disease. Mild and moderate dysplasias (CIN1 and CIN2) usually regress. The spontaneous regression rates for mild dysplasia within 5 years vary between 30% and 90%, whereas the regression rates for moderately dysplastic lesions vary between 20% and 54%.⁹⁻¹² CIN3 and CIS lesions generally tend to progress.

The well-known precursor lesions, the ease with which the organ can be examined, and the known role of these lesions in the development of cancer have made cervical cancer a good candidate for chemoprevention. Nonetheless, since the first trial more than 20 years ago, no important advances have been achieved. The potential for success of chemoprevention is tightly linked to the mechanisms of carcinogenesis. As the carcinogenic mechanisms of cervical cancer become better understood, new, more effective drugs will be available for clinical chemoprevention trials. In this chapter, the current status of chemoprevention in cervical cancer is reviewed.

RISK FACTORS

Various carcinogenic agents, including chemical, physical, and viral, target the genetic material of the cell during carcinogenesis. As a consequence of genetic changes, the proliferative and differentiating control of normal cervical mucosal cells is disrupted. For most cancers, the etiologic agent and exact mechanism of carcinogenesis is not fully understood, although some risk factors are usually known. Many risk factors have also been reported for cervical cancer.

The well-known strong risk factors for cervical cancer include early age at first intercourse (≤ 16 years), a high lifetime number of sexual partners, a history of genital human papillomavirus (HPV) infection or other sexually transmitted diseases, the presence of other genital tract neoplasia, and prior squamous intraepithelial lesions.^{13,14} A number of less relevant risk factors for cervical cancer,

including smoking, immunodeficiency, poor nutrition, human immunodeficiency virus positivity, HPV antibody positivity, intercourse with a sexual partner having risk factors for sexually transmitted disease, long-term use of oral contraceptives, and grand multiparity, have also been reported.¹⁴⁻¹⁶ Although recent findings suggest that HPV is a causative agent for cervical cancer, a small number of patients without documented HPV positivity exist, and the small incidence of cancer as contrasted to the high incidence of HPV infection raises the possibility that the aforementioned risk factors are cofactors. However, recent epidemiological data show that most of the previously well-known risk factors are important for HPV-induced cervical carcinogenesis.¹⁷

Genital HPV infection is one of the most common sexually transmitted diseases. Up to 90% of abnormal cell changes in Pap smears are reportedly related to HPV.^{18,19} As mentioned earlier, whereas most of the low-grade squamous intraepithelial lesions (CIN1)—the most common cytopathologic manifestations of cervical HPV infection—generally regress spontaneously over time, high-grade squamous intraepithelial lesions (CIN2-3) usually persist and may lead to invasive cancer. However, the reasons for development of high-grade squamous intraepithelial lesions and progression to cancer in HPV-infected women are as yet unclear. Among the more than 100 genotypes of HPV, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are classified as high-risk viruses, and infections with these types of HPV are most likely to progress to cancer.^{20,21} HPV16 is the most prevalent type in CIN2-3 and invasive cancers,²¹⁻²³ but it is also the most common type found in women with normal cytologic findings.²¹

CERVICAL CARCINOGENESIS

HPV Infection as the First Step

HPV is now accepted as the primary etiologic agent for cervical cancer. In conjunction with the epidemiological studies that demonstrate the close relationships between HPV infections and cervical cancer, the data on HPV DNA integrated to the cancer cell genome lend strength to the plausibility of HPV's etiologic role.^{24,25} The prevalence of HPV DNA, though dependent on the method used, has been as high as 20%-50% among young women between the ages of 20 to 25.^{25,26} The cohort studies have shown that cervical HPV DNA detection declines with age.²⁷ However, the antibodies against HPV particles increase with age, which indirectly indicates the possible role of humoral immunity in the clearance of virus.²⁷

Most individuals who are exposed to HPV clear the infection on the basis of the cellular and humoral immune response to the viral antigens expressed on the surface of the infected cells.²⁸ Only in a small fraction of women does the HPV infection persist and do lesions induced by the virus in these chronically infected individuals progress to CIN2-3 and, eventually, to invasive cancer. Indi-

viduals who harbor chronic HPV infection may have a cryptic immune response defect that accounts for their inability to clear the chronic infection.²⁸ Genetic or acquired immunosuppression (i.e., human immunodeficiency virus infection) is known to be a cofactor for progression of HPV-induced lesions.^{28,29}

Papillomaviruses are circular, double-stranded DNA viruses that infect the squamous epithelium. The virus, generally spread through sexual contact, enters through a break in the skin and infects the basal cells of the cervical mucosa. The interval between entry of the virus to development of a lesion varies from several weeks to months. After an initial period in which the copy number of HPV episomal DNA increases to 50 copies per cell,³⁰ the copy number stabilizes and, thereafter, the HPV DNA replicates as an episome in synchrony with the replication of the host cell, using the host DNA polymerase. This process is called *latent viral DNA replication*. As the cells in the mucosa mature to the point at which they no longer have the capacity to undergo cellular division, the copy number of the HPV episomal DNA increases dramatically in a manner independent of cellular division.³¹ After this occurs, the late viral genes are expressed and sufficient levels of envelope proteins are produced to promote the packaging of infectious particles. This process is called *vegetative viral DNA replication*.

Carcinogenic Switch: Transition from Chronic Infection and Dysplasia to Invasive Cancer

The HPV viral genome consists of approximately 8 kb, and the protein-coding genes are located on only one of the DNA strands. Within the HPV genome are three distinct regions. The *early region* encodes the viral proteins for viral DNA replication, transcriptional regulation, and cellular transformation. The second distinct region, the *late region*, encodes the viral capsid proteins. The third region, known as the *long control region* (LCR) or *upstream regulatory region*, contains cis-regulatory elements as the origin of the DNA replication and some important transcriptional enhancers.³²

The genes located in the early region are important for HPV-induced lesions. The *E1* and *E2* genes usually take part in the regulation of the LCR. The *E1* gene encodes a viral DNA helicase, which is a DNA-specific binding protein necessary for the initiation of viral DNA synthesis.³³ Likewise, the *E2* gene encodes a DNA binding protein that promotes the binding with host cell DNA polymerase and *E1*. *E2* also regulates the transcription of transforming genes, depending on the level of *E2* and *E2* binding sites within the promoter sequences.³⁴ Both *E6* and *E7* genes of the HPV early region encode cellular transforming proteins. The *E6* oncoprotein binds to several cellular proteins, activating the ubiquitin pathway of proteolysis, which finally targets p53 tumor suppressor protein.³⁵ The *E7* protein of HPV binds pRb and members of the pRB family such as p107 and p130, leading the release of active E2F transcription factor, which finally stimulates cell cycle progression.³⁶ The high- and low-risk HPV viral types mainly differ in

the expression of those transforming genes, the process of genetic integration, and the mechanisms by which the LCR is influenced.³⁷

Once a chronically HPV-infected mucosal surface is established, the *E6* and *E7* HPV genes are expressed sufficiently to reduce the intracellular level of p53 and to functionally inactivate the RB protein. As a consequence, the cervical mucosal cell loses its control of cell cycle progression at the G₁/S restriction point, and genomic instability eventually occurs. In addition, the sensitivity of the cell to DNA damage-induced apoptosis is reduced. This produces an environment in which the probability of acquisition of somatic mutations and chromosomal abnormalities is increased. Thus, a chronically HPV-infected dysplastic mucosa often has chromosomal rearrangements.³⁸ At some point, the HPV DNA episome integrates, often at the site of the HPV *E2* gene, and the integrated HPV DNA, no longer under the restrictive influence of the *E1* or *E2* protein, produces higher levels of *E6* and *E7* expression. The consequent increase of the expression of the *E6* and *E7* genes finally leads to invasive cervical cancer.

The continued replication of the HPV episomal DNA is necessary to maintain chronic infection. The first step in the assembly of a replication initiation complex is the binding of the dimeric *E2* protein to the origin of replication contained within the LCR of the HPV DNA. This is followed by the binding of *E1* (helicase), which then enables the host cell DNA polymerase to bind to the origin of replication. In the absence of *E2*, the *E1* protein cannot bind sufficiently stably to the LCR to initiate the replication initiation complex. The effect of the *E2* gene on gene transcriptional activation at other sites in the HPV DNA may depend in part on the level of *E2* in the cells. At low *E2* levels, HPV *E2* is a transactivator and, at very high levels, HPV *E2* is a transcriptional repressor.^{39,40} Both *E1* and *E2* are necessary for papilloma virus formation induced by papillomavirus genes.³⁴

CERVICAL CANCER CHEMOPREVENTION TRIALS

Endpoints for Chemoprevention Trials

Chemoprevention, the method of inhibiting carcinogenesis before the invasive cancer is established, has attracted a great deal of attention thus far. Chemoprevention trials in cancer have two major endpoints. The first is, in the clinical setting, to prevent or delay the development of clinical cancer. Although this is the main goal of chemoprevention trials, it is not useful as an endpoint for clinical chemoprevention trials. To achieve the primary endpoint of reducing cervical cancer incidence, large, long-term, randomized studies are required. Managing and conducting such studies is extremely difficult, because they involve a large number of patients and require years of follow up. The natural history of cervical cancer has already been outlined. The existence of effective treatment modalities for patients with CIN3, which constitutes the optimal target group for chemoprevention, is likely to lead to problems with recruitment for and compliance with prevention trials.

Instead of using the ultimate goal of reducing cervical cancer incidence as an endpoint, biomarkers may serve as intermediate or surrogate endpoints for chemoprevention trials. Surrogate endpoint biomarkers (SEBs) are useful for randomized phase II trials, which are usually preferred for cervical chemoprevention. Such a marker should be on the pathway to carcinogenesis and ideally should change as the process progresses. Aside from their sensitivity and specificity, potential chemopreventive drugs are chosen on the basis of their ability to modulate the surrogate markers of carcinogenesis. Although not yet validated by large clinical chemoprevention trials, numerous SEBs, such as quantitative cytopathologic markers, proliferation markers such as proliferating cell nuclear antigen, Ki67, cell cycle regulation markers, differentiation markers, genomic instability markers, and tissue maintenance markers, have been used in cervical cancer prevention trials. These SEBs have been well outlined in a recent review.⁴¹ Among these SEBs, cytopathologic markers of altered nuclear features and tissue architecture have been widely used in clinical trials.^{42,43} These markers have also been used as the inclusion criteria of almost all of trials conducted thus far.

The apparent role of viral carcinogenesis in cervical cancer relates the SEBs to HPV. Among those markers used previously, histopathologic nuclear features, HPV viral load, mitotic frequency (MPM-2 antibody staining), proliferating cell nuclear antigen, epidermal growth factor receptor, and aneuploidy demonstrated by flow cytometry are promising factors for cervical chemoprevention trials.⁴³⁻⁴⁶

Chemopreventive Agents and Clinical Trials

Study design in chemoprevention trials is somewhat problematic. After the toxicity tests in Phase I trials have been conducted, which sometimes also evaluate the activity of a given drug in high-risk patients, the activity of the drug is tested in a short-term Phase IIa trial, which also evaluates the predictability of endpoint biomarkers. After an effective drug and useful SEBs are available, definitive randomized Phase IIb trials are conducted. In some studies, Phase II and I designs have been overlapped. Phase III trials should include a large number of patients and aim at long-term efficacy in decreasing cancer incidence. An ideal Phase III trial, therefore, requires thousands of patients and usually more than 10 years of follow up. However, the clinical trials conducted to date for cervical cancer chemoprevention have been mostly randomized Phase IIb or small Phase III studies that included a mere few hundred patients (Table 13.1.1). Therefore, from a statistical perspective, the power of such studies is usually low.

Another important problem encountered in cervical chemoprevention trials is patient selection criteria. Cytopathologic markers, the most widely used SEBs at the tissue level, generally are used to select the target population for clinical chemoprevention trials in cervical carcinoma. Most previous studies aimed to reverse the premalignant lesion. All grades of cervical premalignant lesions have been included in chemoprevention trials (see Table 13.1.1). The results of these

TABLE 13.1.1 Randomized (Phase II-III) Clinical Trials of Cervical Cancer Chemoprevention

11	CIN1-3	32	278/369	de Vet et al ⁸⁸ (1991)
66	CIN1-2	—	47/78	Butterworth et al ⁸⁸ (1992)
27	CIN2	—	232/301	Meyskens et al ⁸⁵ (1994)
31	CIN3	—	246/331	Chikliders et al ⁹⁰ (1995)
6	CIN1-2	—	111/117	Fahey et al ⁸⁹ (1996)
—	CIN2	60	69/98	Romey et al ⁷⁰ (1997)
47	CIN1-3	50		

trials should be reviewed with regard to the spontaneous regression rates, which are as high as 90% in CIN1 lesions. The higher spontaneous regression rate of CIN1-CIN2 lesions has often caused previous trials to fail to show any beneficial effect. Hence, because of the natural history of the disease, patients with severe dysplasia (CIN3) lesions appear to be reasonable candidates for cervical chemoprevention trials.

Many effective drugs have been tested *in vitro* as treatment for cervical lesions. However, the toxic effects of some of those drugs prevent their use in a prevention trial, which usually requires long-term administration of the drug. Also, because the majority of women being targeted for the cervical cancer chemoprevention trials are of reproductive age, use of the retinoids, which have been shown to be effective in some other solid tumor prevention trials, is problematic.

In randomized cervical cancer prevention trials, micronutrients such as folic acid, β -carotene, retinoids, and indole compounds have been used thus far (see Table 13.1.1).

Micronutrients Case-control studies investigating the effects of micronutrients on cervical cancer risk have shown that high intake of dietary carotene, vitamins C and E, and folate are associated with reduced risk.^{47,48} Likewise, the epidemiological studies suggest an increased risk for CIN and cervical cancer with low vitamin A intake.⁴⁹ However, most of these studies investigating the relation between micronutrients and cervical cancer risk were conducted before reliable testing methods for HPV were available. Recent studies of the role of diet in cervical cancer risk have usually taken into account the HPV association. Although an association between HPV persistence and micronutrients has been reported,⁵⁰ the more recent studies report conflicting results with regard to a protective role of micronutrients.^{51,52} In addition, recent reports do not support an association between folate and HPV infection.^{53,54} Among the micronutrients investigated thus far, folic acid and β -carotene have been used in clinical chemoprevention trials.

Although results are conflicting with regard to the association of folate levels and cervical cancer risk, the rationale for folic acid to be used in clinical trials is based mainly on its role in DNA synthesis, repair, and methylation.^{55,56} However, the randomized folate trials published to date reported negative results for its efficacy in CIN.

An early study by Butterworth et al⁵⁷ reported a significantly increased response rate for folic acid, 10 mg/day for 3 months, as compared to placebo (vitamin C, 10 mg) in patients using oral contraceptives.⁵⁷ However, the second largest study of the same group did not show any beneficial treatment effect of the same drug at the same dose in low-income women.⁵⁸ In the second study, involving patients with CIN1-CIN2 lesions, the spontaneous regression rate was 66%, which constituted one of the leading causes of failure of the trial. The beneficial effect seen in the first study might be related to the reversal effects of folic acid on megablastic

TABLE 13.1.1 Continued

Author (Year)	Study Design	Intervention	CIN1	CIN2-3	CIN2-3	CIN3	Overall	P-value
			Number	Number	Number	Number	Number	
Ruidi et al ⁵⁹ (1997)	74/74	Retinamide II, 10 mg, vs placebo, suppository, 6 mo	141/147	27/30	103/124	47	0	0.05 for overall response.
Mackerras et al ⁷¹ (1999)	141/147	β -Carotene, 30 mg, vs placebo	27/30	103/124	47	0	0	0.05 for overall response.
Bell et al ¹⁰⁰ (2000)	27/30	Indole-3-carbinol, 200 mg, PO, 6 mo vs placebo	103/124	47	0	0	0	0.05 for overall response.
Keefe et al ⁷² (2001)	103/124	β -Carotene, 30 mg, vs placebo, PO, 24 mo	47	0	0	0	0	0.05 for overall response.

CIN, cervical intraepithelial neoplasia; PO, per os (orally).

*P < .05 for only complete response.

*P < .05 for overall response.

changes seen in women using oral contraceptives. The lack of a relationship between folic acid and HPV makes unlikely a reversing effect for this nutrient in HPV-induced lesions of cervical mucosa.⁵⁹ In the Phase III trial conducted by the Southwest Oncology Group,⁶⁰ folic acid was used at a dose of 5 mg/day for 6 months in patients with CIN1-CIN2 lesions. Again, the regression rates of lesions assessed by Pap smear and colposcopy did not differ between folic acid and placebo groups.

Although the aforementioned trials suffer from several limitations—short duration of the intervention and recruiting of patients with CIN1-CIN2 lesions, which have high spontaneous regression rates—it is unfavorable results have prevented the use of folic acid in further trials. Nevertheless, testing of this agent in combination with other drugs for chemoprevention might be warranted on the basis of folic acid's known effects on DNA synthesis and methylation.

β -Carotene is a precursor of vitamin A (retinol). Other than being a provitamin A, it exhibits a broad range of activities, such as modulation of immune response, inhibition of viral gene expression, and antioxidant activity.⁶¹⁻⁶³ Epidemiological studies have shown an inverse relationship between β -carotene levels and cervical cancer and CIN risk.^{48,64,65} Although a similar relationship has been reported with chronic HPV infection,⁶³ this finding is not supported in other studies.^{66,67} Therefore, the exact role of β -carotene in HPV infection and HPV-induced carcinogenesis remains unclear.

As with the folate trials, the randomized β -carotene trials published thus far have failed to show any beneficial effect of this micronutrient. de Vet et al,⁶⁸ in their randomized trial of β -carotene using 10 mg/day for 3 months, as compared with placebo, have reported similar response rates for both arms. Although this study included CIN1-CIN3 lesions, no significant difference was found with respect to the degree of dysplasia after treatment. In another randomized trial, Fairley et al⁶⁹ were unable to show a beneficial effect of 30 mg/day β -carotene for 12 months on the lesions of CIN1-CIN2. On the contrary, Romney et al⁷⁰ reported a significantly higher complete remission rate for the placebo arm as compared to the β -carotene arm of a trial involving administration of 30 mg/day for 9 months, although the overall response rates of the placebo and drug groups were similar. Moreover, no significant relationship between plasma β -carotene levels and regression of CIN was found in that trial. In the trial by Mackerras et al,⁷¹ patients were randomized among three arms: β -carotene only, 30 mg/day; β -carotene plus vitamin C, 500 mg/day; and placebo, for 6 months. Although not significant, the combination arm had the worst response rate. In the very recent Phase III study by Keefe et al,⁷² even the prolonged use of β -carotene, 30 mg/day for 24 months, did not enhance the regression rates of CIN2-CIN3 lesions, especially in patients positive for human immunodeficiency virus. The outlined results of these randomized studies suggest no beneficial treatment effect of β -carotene in cervical cancer chemoprevention.

Retinoids Retinoids are potent modulators of epithelial cell growth and differentiation. They have been reported to inhibit cell proliferation and to induce cell differentiation.⁷³ Both the metabolites of vitamin A and its synthetic analogs binding retinoid receptors are the most effective drugs studied thus far in solid tumor chemoprevention trials. Two main types of retinoid receptors in the nucleus have been identified. Whereas the first identified receptor type, retinoic acid receptors α , β , and γ , binds both all-*trans*-retinoic acid and *cis*-retinoic acid, the second group of receptors, retinoid X receptors α , β , and γ , binds only 9-*cis*-retinoic acid.^{74,75} The studies on tissues from both premalignant and malignant lesions suggest that the decreased expression of retinoid receptors might be related to tumor promotion.⁷⁶ Also, the reduced expression of retinoic acid receptors α , β , and γ has been reported in CIN1-CIN3 lesions.⁷⁷ As the molecular aspects of retinoid receptors become clearer, the number of new synthetic analogs of retinol may increase.

The exact mechanisms of the antiproliferative effect of retinoids on HPV-induced cells are not well understood. Although in a clinical report 13-*cis*-retinoic acid has been shown to decrease the titer of high-risk HPVs, its molecular effects on HPV-induced transformation are potentially more important.⁷⁸ Earlier studies have reported that the suppressive effects of retinoic acid on HPV16-transformed cells might be related to the inhibition of E6/E7 oncoproteins.^{79,80} Recent studies show that besides their E6/E7 inhibitory effects, retinoids may induce the expression of p53 in HPV-infected cells, which might partially reverse the E6-induced degradation of this tumor suppressor protein.⁸¹ Also, the growth inhibitory effects of these compounds on HPV-induced lesions were found to be mediated by transforming growth factor- β , which seems to augment the HPV-induced decrement of both p53- and pRb-dependent functions.^{81,82} This finding suggests that the loss of retinoid sensitivity seen in the later phases of HPV-induced carcinogenesis, shown *in vitro* and *in vivo*, might be related to transforming growth factor- β -mediated resistance. Further studies demonstrated that the increasing effects of retinoids on insulinlike growth factor binding proteins 3 and 5 levels also contribute to antiproliferative effect.^{83,84} These many effects of retinoids underscore the importance of these compounds in cervical cancer chemoprevention trials.

Hundreds of retinoic acid receptor and retinoid X receptor ligands have been synthesized and tested. However, their important side effects, especially the teratogenic effects, limit the use of these compounds in cervical chemoprevention trials. Because most patients in the target population for cervical cancer chemoprevention studies are of reproductive age (unlike the women who compose the population for other solid tumor prevention studies), no randomized clinical trial using systemic administration of retinoids has been published thus far. In lieu of systemic administration, topical application has been widely used. In the first Phase III cervical cancer prevention trial of retinoids, Meyskens et al⁸⁵ reported a significant regression of CIN2 lesions with topical use of *b-trans*-retinoic acid as a 0.375%

cream. However, no significant response was found for CIN3 lesions. In a recent interim analysis of a randomized study from China, retinamide II suppositories were shown to incite a significant therapeutic effect on CIN1-CIN2 lesions.⁸⁶ Further randomized trials will determine the exact role of topical retinoids in cervical cancer prevention.

The systemic administration of retinoid compounds in the chemoprevention of other solid tumors has yielded positive results.⁸⁷ However, as mentioned earlier, the toxicity of these drugs limits their systemic use in patients with CIN lesions. Trials with new and less toxic retinoid compounds, as in the ongoing trial of fenretidine [N-(4-hydroxyphenyl) retinamide] by Fullen et al,⁸⁸ will determine the efficacy of oral administration of these compounds.

Indole Compounds Many vegetables and fruits, which are found on the American Cancer Society's daily recommended diet for cancer prevention, include indole compounds. Indole-3-carbinol, one of these indole compounds, has been approved by the U.S. Food and Drug Administration as a nutritional supplement. No significant toxicity of this compound has been reported.⁸⁹ Indole-3-carbinol has been reported to exert various indirect and direct inhibitory effects on carcinogenesis. Its indirect effects include induction of the detoxification action of cytochrome P450,⁹⁰ reduction of the activation of carcinogens by binding to the aryl hydrocarbon receptor,⁹¹ and induction of C2 hydroxylation of estrogen, which may play an additive role in HPV-induced carcinogenesis of the cervical mucosa.⁹² The direct effects include induction of apoptosis in some tumor cell lines (e.g., MCF-7 breast cancer cells)⁹³ and of cell cycle arrest at G₁/S restriction points by inhibition of cyclin-dependent kinase 6.⁹⁴ In a cottontail rabbit papillomavirus animal model, Wu et al⁹⁵ reported that while the wild-type cottontail rabbit papillomavirus genome, intradermally inoculated within a gene gun, induced papilloma formation on inoculation sites, no papillomas were seen with frame shift mutations into the E1 and E2 genes of the cottontail rabbit papillomavirus genome. Therefore, drugs that could inhibit the functions of E1 and E2 in promoting both the latent and vegetative replication of HPV could eradicate HPV infections in individuals unable to clear such infections and might be used as chemopreventive agents. Indole compounds have recently been shown to inhibit the binding of proteins to E2.⁹⁶ In a mouse model, indole-3-carbinol has been shown to inhibit HPV-induced cervical carcinogenesis.⁹⁷

Indole-3-carbinol also has been tested and proven successful as a treatment of HPV11-associated laryngeal papillomatosis in a few children⁹⁸ and in 18 adults with recurrent HPV11-related laryngeal papillomatosis.⁹⁹ This experience showed a 30% response rate and 30% stabilization of disease rate in the involved patients. In a recent small randomized trial of indole-3-carbinol by Bell et al,¹⁰⁰ a 50% complete remission rate in patients with CIN2-CIN3 lesions has been reported. Nonetheless, further randomized trials are required to establish the efficacy of indole compounds in cervical cancer prevention. A randomized, double-blind

trial of indole-3-carbinol in cervical dysplasia sponsored by a grant from the NIH is about to begin.

Other Chemopreventive Agents

Some other agents also are under investigation for possible chemopreventive effects. A strong correlation between the levels of polyamines and cell proliferation has led to the synthesis of the inhibitors of ornithine decarboxylase enzyme, a key enzyme in polyamine biosynthesis. Difluoromethylornithine, a specific inhibitor of ornithine decarboxylase, has already been used in chemoprevention trials of some solid tumors. However, its high potential for toxicity, especially ototoxicity, has limited its use.¹⁰¹ Nonetheless, in a Phase I trial of this drug in patients with CIN3 lesions, Mitchell et al¹⁰² reported no significant toxicity. This same research group has already completed patient accrual for a Phase II study of efloornithine in patients with CIN2-CIN3 lesions.¹⁰³ Further trials are needed to establish the chemopreventive role of polyamine synthesis inhibitors in cervical cancer.

FUTURE CONSIDERATIONS

Although the availability of effective screening programs in some countries has already decreased its incidence and mortality, cervical cancer continues to be the second leading cause of cancer death among women. Chemoprevention trials for cervical cancer have been disappointing so far. With our increased understanding of the mechanisms that lead to cervical cancer, the processing, designing, synthesizing, and testing of new and promising drugs has begun. However, these trials are still in their infancy. The role of HPV in cervical carcinogenesis has also led to vaccine trials, but this research too is in its beginning stages. Along with the chemoprevention and vaccine strategies, risk avoidance and new, efficient early detection technologies may help to decrease the incidence and mortality of cervical cancer.

ACKNOWLEDGMENTS

The authors recognize support from the Bush Leukemia Research Foundation, support from the National Cancer Institute for Dr. Hakan Akbulut's fellowship in the Deisseroth laboratory, and a grant to AD for a prevention trial to evaluate the use of indole-3-carbinol to clear chronic HPV infections.

REFERENCES

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;80:827-841.

2. Greenlee RT, Hill-Harmon MB, Murray T et al. Cancer statistics, 2001. *CA: Cancer J* 2001;51(1): 15-36.
3. Vizcaino AP, Moreno V, Bosch FX et al. International trends in incidence of cervical cancer: II. Squamous-cell carcinoma. *Int J Cancer* 2000;86:429-435.
4. Franco EL, Duarte-Franco E, Ferenczy A. Cervical cancer: epidemiology, prevention and the role of human papillomavirus infection. *CMAJ* 2001;164:1017-1025.
5. Hwang YY, Moon H, Cho SH et al. Ten-year survival of patients with locally advanced, stage Ib-IIb cervical cancer after neoadjuvant chemotherapy and radical hysterectomy. *Gynecol Oncol* 2001;82:88-93.
6. Brewster WR, Monk BJ, Ziegas A et al. Intent-to-treat analysis of stage Ib and IIa cervical cancer in the United States: radiotherapy or surgery 1988-1995. *Obstet Gynecol* 2001;97:248-254.
7. Richart RM. A modified terminology for cervical intraepithelial neoplasia. *Obstet Gynecol* 1990; 75:131-133.
8. National Cancer Institute Workshop. The 1988 Bethesda System for reporting cervical /vaginal cytological diagnosis. *JAMA* 1989;262:931-934.
9. Naisseil K, Roger V, Naisseil M. Behaviour of mild cervical dysplasia during long-term follow-up. *Obstet Gynecol* 1986;67:665-669.
10. Osor AG. Natural history of cervical intraepithelial neoplasia: a critical review. *Int J Gynecol Pathol* 1993;12:186-192.
11. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999;91:252-258.
12. Naisseil K, Naisseil M, Vachlavinkova V. Behavior of moderate cervical dysplasia during long-term follow-up. *Obstet Gynecol* 1983;61:609-614.
13. Schiffman MH, Brinton LA. The epidemiology of cervical carcinogenesis. *Cancer* 1995;76: 1888-1901.
14. Kjellberg L, Hallmans G, Ahren AM et al. Smoking, diet, pregnancy and oral contraceptive use as risk factors for cervical intra-epithelial neoplasia in relation to human papillomavirus infection. *Br J Cancer* 2000;82:1332-1338.
15. Lacey JV Jr, Frisch M, Brinton LA et al. Associations between smoking and adenocarcinomas and squamous cell carcinomas of the uterine cervix (United States). *Cancer Causes Control* 2001;12:153-161.
16. Tjiong MY, Out TA, Ter Schegget T et al. Epidemiologic and mucosal immunologic aspects of HPV infection and HPV-related cervical neoplasia in the lower female genital tract: a review. *Int J Gynecol Cancer* 2001;11:9-17.
17. Daling JR, Madeleine MM, McKnight B et al. The relationship of human papillomavirus-related cervical tumors to cigarette smoking, oral contraceptive use and prior herpes simplex virus type 2 infection. *Cancer Epidemiol Biomarkers Prevent* 1996;5:541-548.
18. Schiffman MH, Bauer HM, Hoover RN et al. Epidemiological evidence showing that human papilloma virus infection causes most cervical intraepithelial neoplasia. *J Natl Cancer Inst* 1993; 85:958-964.
19. Herrero R, Hildesheim A, Bratti C et al. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. *J Natl Cancer Inst* 2000;92:464-474.
20. Ho GY, Bierman R, Beardsley L et al. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med* 1998;338:423-428.
21. Ngangel C, Munoz N, Bosch FX et al. Causes of cervical cancer in the Philippines: a case-control study. *J Natl Cancer Inst* 1998;90:43-49.
22. Woodman CB, Collins S, Winter H et al. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet* 2001;357:1831-1836.
23. Berumen J, Ordonez RM, Lazcano E et al. Asian-American variants of human papillomavirus 16 and risk for cervical cancer: a case-control study. *J Natl Cancer Inst* 2001;93:1325-1330.
24. Bosch FX, Manos MM, Munoz N et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst* 1995;87:796-802.
25. Rohan T, Mann V, McLaughlin J et al. PCR-detected genital papillomavirus infection: prevalence and association with risk factors for cervical cancer. *Int J Cancer* 1991;49:1-5.
26. Moscicki AB, Hills N, Shiboski S et al. Risks for incident human papillomavirus infection and low-grade squamous intraepithelial lesion development in young females. *JAMA* 2001;285: 2995-3002.
27. Bonkles HJ, de Grijl TD, Walboomers JM et al. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia: II. Systemic but not local IgA responses correlate with clearance of HPV-16. *J Gen Virol* 1999;80(2):409-417.
28. Sillman F, Stanek A, Sedlis A et al. The relation between human papilloma virus infection and lower genital intraepithelial neoplasia in immunosuppressed women. *Am J Obstet Gynecol* 1984; 150:300-308.
29. Palefsky JM, Minkoff H, Kalish LA et al. Cervicovaginal human papillomavirus infection in human immunodeficiency virus-1 (HIV)-positive and high-risk HIV- negative women. *J Natl Cancer Inst* 1999;91:226-236.
30. Schneider A, Oltersdorf T, Schneider V et al. Distribution of human papilloma virus 16 genome in cervical neoplasia by molecular in situ hybridization of tissue sections. *Int J Cancer* 1987; 39:717-723.
31. Meyers C, Wetstern FL. The late region differentially regulates the in vitro transformation by cottontail rabbit papilloma virus DNA in different cell types. *Virology* 1991;181:637-646.
32. Howley PM, Lowy DR. Papillomaviruses and their replication. In: Knipe DM, Howley PM, eds. *Fields Virology*, vol 2. Philadelphia: Lippincott Williams & Wilkins, 2001:2197-2229.
33. Wilson WG, Ludes-Meyers J. A bovine papilloma virus E1-related protein binds specifically to bovine papillomavirus DNA. *J Virol* 1991;65:5314-5322.
34. Wu X, Zial W, Brandsma JL. Papilloma formation by cottontail rabbit virus requires E1 and E2 regulatory genes in addition to E6 and E7 transforming genes. *J Virol* 1994;68:6097-6102.
35. Scheffner M, Werness BA, Huibregtse JM et al. The E6 oncoprotein encoded by human papillomavirus type s 16 and 18 promotes the degradation of p53. *Cell* 1990;63:1129-1136.
36. Phillips AC, Vousden KH. Human papillomavirus and cancer: the viral transforming genes. *Cancer Surv* 1999;33:55-74.
37. Barbosa MS, Vass WC, Lowy DR, Schaller JT. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. *J Virol* 1991;65:292-298.
38. Atkin MB, Baker MC. Nonrandom chromosome changes in carcinoma of the cervix uteri: II. Ten tumors in the triploid-tetraploid range. *Cancer Genet Cytogenet* 1984;13:18-27.
39. Sverdrup FM, Sheahan LC, Khan SA. Development of human papilloma virus plasmids capable of episomal replication in human cell lines. *Gene Ther* 1999;6:1317-1321.
40. Fujii T, Brandsma JL, Peng XY et al. Species specific mechanisms of transcriptional regulation by cottontail rabbit papillomavirus and human papillomavirus 16. *J Biol Chem* 2001;276: 867-874.
41. Foller M, Schottenfeld D. Surrogate end-point biomarkers and their modulation in cervical chemoprevention trials. *Cancer* 2001;91:1758-1776.

42. Sun CA, Lai HC, Chang CC et al. The significance of human papillomavirus viral load in prediction of histologic severity and size of squamous intraepithelial lesions of uterine cervix. *Gynecol Oncol* 2001;83:95-99.
43. Hu W, Mitchell MF, Boiko IV et al. Progressive dysregulation of proliferation during cervical carcinogenesis as measured by MPM-2 antibody staining. *Cancer Epidemiol Biomarkers Prev* 1997;6:711-718.
44. Shurbaji MS, Brooks SK, Thurmond TS. Proliferating cell nuclear antigen immunoreactivity in cervical intraepithelial neoplasia and benign cervical epithelium. *Am J Clin Pathol* 1993;100:22-26.
45. Boiko IV, Mitchell MF, Hu W et al. Epidermal growth factor receptor expression in cervical intraepithelial neoplasia and its modulation during an a-difluoromethylornithine chemoprevention trial. *Clin Cancer Res* 1998;4:1383-1391.
46. Lai CH, Hsueh S, Tsao KC, Chen HM. DNA aneuploidy in benign tumors and normal tissues of the female genital tract. *Int J Gynecol Pathol* 1996;15:63-68.
47. Ziegler RG, Brinton LA, Hamman RF et al. Diet and the risk of invasive cervical cancer among white women in the United States. *Am J Epidemiol* 1990;132:432-445.
48. Pottschman N, Brinton LA. Nutrition and cervical neoplasia. *Cancer Causes Control* 1996;7:113-126.
49. La Vecchia C, Decarli A, Fasoli M et al. Dietary vitamin A and the risk of intraepithelial and invasive cervical neoplasia. *Gynecol Oncol* 1988;30:187-195.
50. Giuliano AR, Papenfuss M, Nour M et al. Antioxidant nutrients: association with persistent human papillomavirus infection. *Cancer Epidemiol Biomarkers Prev* 1997;6:917-923.
51. Wideroff L, Pottschman N, Glass AG et al. A nested case-control study of dietary factors and the risk of incident cytological abnormalities of the cervix. *Nutr Cancer* 1998;30:130-136.
52. Goodman MT, McDuffie K, Hernandez B et al. Case-control study of plasma folate, homocysteine, vitamin B(12), and cysteine as markers of cervical dysplasia. *Cancer* 2000;89:376-382.
53. Fowler BM, Giuliano AR, Pyattlake C et al. Hypomethylation in cervical tissue: Is there a correlation with folate status? *Cancer Epidemiol Biomarkers Prev* 1998;7:901-906.
54. Weinstein SJ, Ziegler RG, Frongillo EA Jr et al. Low serum and red blood cell folate are moderately, but insignificantly associated with increased risk of invasive cervical cancer in U.S. women. *J Nutr* 2001;131:2040-2048.
55. Alberg AJ, Shelhub J, Shah KV et al. The risk of cervical cancer in relation to serum concentrations of folate, vitamin B12 and homocysteine. *Cancer Epidemiol Biomarkers Prev* 2000;9:761-764.
56. Mason JB, Levesque T. Folate: the effects on carcinogenesis and the potential for cancer chemoprevention. *Oncology* 1996;10:1727-1742.
57. Butterworth CE Jr, Hatch KD, Gore H et al. Improvement in cervical dysplasia associated with folic acid therapy in users of oral contraceptives. *Am J Clin Nutr* 1982;35:73-82.
58. Butterworth CE Jr, Hatch KD, Soong SF et al. Oral folic supplementation for cervical dysplasia: a clinical intervention trial. *Am J Obstet Gynecol* 1992;166:803-809.
59. Weinstein SJ, Ziegler RG, Frongillo EA Jr et al. Low serum and red blood cell folate are moderately, but insignificantly associated with increased risk of invasive cervical cancer in U.S. women. *J Nutr* 2001;131:2040-2048.
60. Childers JM, Chu J, Voigt LF et al. Chemoprevention of cervical cancer with folic acid: a phase II Southwest Oncology Group Intergroup study. *Cancer Epidemiol Biomarkers Prev* 1995;4:155-159.
61. Rock CL, Michael CW, Reynolds RK, Ruffin MT. Prevention of cervix cancer. *Crit Rev Oncol Hematol* 2000;33:169-185.
62. Gossage C, Deyhim M, Moser-Veillon PB et al. Effect of beta-carotene supplementation and lactation on carotenoid metabolism and mitogenic T lymphocyte proliferation. *Am J Clin Nutr* 2000;71:950-955.
63. Schiff MA, Patterson RE, Baumgartner RN et al. Serum carotenoids and risk of cervical intraepithelial neoplasia in southwestern American Indian women. *Cancer Epidemiol Biomarkers Prev* 2001;10:1219-1222.
64. Palan PR, Mikhail MS, Goldberg GL et al. Plasma levels of beta-carotene, lycopene, canthaxanthin, retinol, and alpha- and tau-tocopherol in cervical intraepithelial neoplasia and cancer. *Clin Cancer Res* 1996;2:181-185.
65. Giuliano AR, Papenfuss M, Nour M et al. Antioxidant nutrients: association with persistent human papillomavirus infection. *Cancer Epidemiol Biomarkers Prev* 1997;6:917-923.
66. Tabrizi SN, Fairley CK, Chen S et al. Epidemiological characteristics of women with high grade CIN who do and do not have human papillomavirus. *Br J Obstet Gynaecol* 1999;106:252-257.
67. Nagata C, Shimizu H, Yoshikawa H et al. Serum carotenoids and vitamins and risk of cervical dysplasia from a case-control study in Japan. *Br J Cancer* 1999;81:1234-1247.
68. de Vet HC, Knipschild PG, Willebrand D et al. The effect of beta-carotene on the regression and progression of cervical dysplasia: a clinical experiment. *J Clin Epidemiol* 1991;44:273-283.
69. Fairley CK, Tabrizi SN, Chen S et al. A randomized clinical trial of beta carotene vs placebo for the treatment of cervical HPV infection. *Int J Gynecol Cancer* 1996;6:225-230.
70. Romney SL, Ho GY, Palan PR et al. Effects of b-carotene and other factors on outcome of cervical dysplasia and human papillomavirus infection. *Gynecol Oncol* 1997;65:483-492.
71. Mackerras D, Irwig L, Simpson JM et al. Randomized double-blind trial of beta-carotene and vitamin C in women with minor cervical abnormalities. *Br J Cancer* 1999;79:1448-1453.
72. Keefe KA, Schell MJ, Brewer C et al. A randomized, double blind, phase III trial using oral beta-carotene supplementation for women with high-grade cervical intraepithelial neoplasia. *Cancer Epidemiol Biomarkers Prev* 2001;10:1029-1035.
73. Yokoyama M, Nakao Y, Iwasaka T et al. Retinoic acid and interferon-alpha effects on cell growth and differentiation in cervical carcinoma cell lines. *Obstet Gynecol* 2001;98:332-340.
74. Allenby G, Janoch R, Kazmer S et al. Binding of 9-cis retinoic acid and all-trans retinoic acid to retinoic acid receptor α , β and γ . *J Bio Chem* 1994;269:16689-16695.
75. Narayanan BA, Holladay EB, Nixon DW, Mauro CT. The effect of all-trans and 9-cis retinoic acid on the steady state level of HPV16 E6/E7 mRNA and cell cycle in cervical carcinoma cells. *Life Sci* 1998;63:565-573.
76. Xu X-C, Ro JY, Lee JS et al. Differential expression of nuclear retinoic acid receptors in normal, premalignant and malignant head and neck tissues. *Cancer Res* 1994;54:3580-3587.
77. Xu XC, Mitchell MF, Silva E et al. Decreased expression of retinoic acid receptors, transforming growth factor beta, involucrin, and cornifin in cervical intraepithelial neoplasia. *Clin Cancer Res* 1999;5:1503-1508.
78. Ahn WS, Lee JM, Namkoong SE et al. Effect of retinoic acid on HPV titration and colposcopic changes in Korean patients with dysplasia of the uterine cervix. *J Cell Biochem Suppl* 1997;28/29:133-139.
79. Creek KE, Jenkins GR, Khan MA et al. Retinoic acid suppresses human papillomavirus type 16 (HPV16)-mediated transformation of human keratinocytes and inhibits the expression of the HPV16 oncogenes. *Adv Exp Med Biol* 1994;354:19-35.
80. Khan MA, Jenkins GR, Tolleson WH et al. Retinoic acid inhibition of human papillomavirus type 16-mediated transformation of human keratinocytes. *Cancer Res* 1993;53:905-909.
81. Nees M, Geoghegan JM, Munson P et al. Human papillomavirus type 16 E6 and E7 proteins

- inhibit differentiation dependent expression of transforming growth factor $\beta 2$ cervical keratinocytes. *Cancer Res* 2000;60:4289-4298.
82. Boyger DR, Mi Y, Geslani G et al. Retinoic acid resistance at late stages of human papillomavirus type 16-mediated transformation of human keratinocytes arises despite intact retinoid signaling and is due to a loss of sensitivity to transforming growth factor-beta. *Virology* 2000;270:397-407.
 83. Hembree JR, Agarwal C, Beard RL et al. Retinoid X receptor-specific retinoids inhibit the ability of retinoic acid receptor-specific retinoids to increase the level of insulin-like growth factor binding protein-3 in human ectocervical epithelial cells. *Cancer Res* 1996;56:1794-1799.
 84. Higo H, Duan C, Clemmons DR, Herman B. Retinoic acid inhibits cell growth in HPV negative cervical carcinoma cells by induction of insulin-like growth factor binding protein-5 (IGFBP-5) secretion. *Biochem Biophys Res Commun* 1997;239:706-709.
 85. Meykens FL Jr, Surwit E, Moon TE et al. Enhancement of regression of cervical intraepithelial neoplasia II (moderate dysplasia) with topically applied all-trans-retinoic acid: a randomized trial. *J Natl Cancer Inst* 1994;86:539-543.
 86. Ruidi C, Alhua D, Pelyu B et al. Chemoprevention of cancer of uterine cervix: a study on chemoprevention of retinamide II from cervical precancerous lesions. *J Cell Biochem Suppl* 1997;28(29):140-143.
 87. Hansen LA, Sigman CC, Andreola F et al. Retinoids in chemoprevention and differentiation therapy. *Carcinogenesis* 2000;21:1271-1279.
 88. National Cancer Institute PDQ. Phase III randomized, double blinded, placebo controlled study of fenretidine in patients with cervical intraepithelial neoplasia (CIN) grade 2-3. (Protocol ID No. NCI-P97-0092). *Curr Clin Trials Oncol* 2001;
 89. Bradlow HL, Michnovicz JJ, Harper M et al. Long term responses of women to indole or a high fiber diet. *Cancer Epidemiol Biomarkers Prev* 1994;3:591-595.
 90. Fong AT, Swanson HI, Dashwood RH et al. Mechanisms of anticarcinogenesis by indole-3-carbinol: studies of enzyme induction, electrophile scavenging, and inhibition of aflatoxin B activation. *Biochem Pharmacol* 1990;39:19-26.
 91. Chen I, Safe S, Bjaldenes L. Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. *Biochem Pharmacol* 1996; 51:1069-1076.
 92. Michnovicz JJ, Aldercreutz H, Bradlow HL. Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. *J Natl Cancer Inst* 1997;89:718-723.
 93. Ge XK, Yannal S, Rennett G et al. 3,3'-Diindolylmethane induces apoptosis in human cancer cells. *Biochem Biophys Res Commun* 1996;228:153-158.
 94. Cover CM, Hsieh SJ, Tran SH et al. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. *J Biol Chem* 1998;273:3838-3847.
 95. Wu X, Zial W, Brandsma JL. Papilloma formation by cottontail rabbit virus requires E1 and E2 regulatory genes in addition to E6 and E7 transforming genes. *J Virol* 1994;68:6097-6102.
 96. Webster K, Taylor A, Gaston K. Oestrogen and progesterone increase the levels of apoptosis induced by the human papillomavirus type 16 E2 and E7 proteins. *J Gen Virol* 2001;82(1): 201-213.
 97. Jin L, Qi M, Chen D-Z et al. Indole-3-carbinol prevents cervical cancer in human papilloma virus type 16 (HPV16) transgenic mice. *Cancer Res* 1999;59:3991-3997.
 98. Coll DA, Auburn K, Pocsic WP, Bradlow HL. Treatment of recurrent respiratory papillomatosis with indole-3-carbinol. *Am J Otolaryngol* 1997;18:1-4.
 99. Rosen CA, Wopodson GE, Thompson JWQ et al. Preliminary results of the use of indole-3-carbinol for recurrent respiratory papillomatosis. *Otolaryngol Head Neck Surg* 1998;118: 910-915.
 100. Bell MC, Crowley-Nowick P, Bradlow JL et al. Placebo-controlled trial of indole-3-carbinol in the treatment of CIN. *Gynecol Oncol* 2000;78:123-129.
 101. Love RR, Jacoby R, Newton MA et al. A randomized, placebo-controlled trial of low-dose alpha-difluoromethylornithine in individuals at risk for colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 1998;7:989-992.
 102. Mitchell ME, Tortolero-Luna G, Lee JJ et al. Phase I dose de-escalation trial of alpha-difluoromethylornithine in patients with grade 3 cervical intraepithelial neoplasia. *Clin Cancer Res* 1998;4: 303-310.
 103. National Cancer Institute PDQ. Phase II randomized study of efloornithine versus placebo in patients with cervical intraepithelial neoplasia grade 2-3. (Protocol ID No. NCI-P00-0149) *Current Clinical Trials Oncology* 2001.

Progress in Oncology 2002

Edited by

Vincent T. DeVita, Jr.
Yale Cancer Center
New Haven, Connecticut

Samuel Hellman
Department of Radiation Oncology
University of Chicago
Chicago, Illinois

Steven A. Rosenberg
National Cancer Institute
National Institutes of Health
Bethesda, Maryland



JONES AND BARTLETT PUBLISHERS
Sudbury, Massachusetts
BOSTON · TORONTO · LONDON · SINGAPORE

THE EFFICIENCY OF REPLICATION-COMPETENT ADENOVIRAL VECTORS
CARRYING L-PLASTIN PROMOTED CYTOSINE DEAMINASE GENE IN COLON
CANCER

Hakan Akbulut M.D. (Ankara University Faculty of Medicine, Dept. of Med. Oncology,
Ankara, Turkey)

Lixin Zhang M.D. Ph.D. (Sidney Kimmel Cancer Center, San Diego, CA)

Albert Deisseroth M.D. Ph.D. (Sidney Kimmel Cancer Center, San Diego, CA)

Corresponding author: Albert Deisseroth, M.D., Ph.D.,
Sidney Kimmel Cancer Center, 10835 Altman Row, San
Diego, CA 92121,
Phone Number: 848-410-4205.
Fax Number: 858-587-8873
e-mail: adeisseroth@skcc.org

Running title: L-Plastin promoted cytosine deaminase gene therapy

Key Words: adenoviral vectors, colon cancer, L-plastin, cytosine deaminase, 5-
fluorocytosine

ABSTRACT

Pro-drug activating transcription unit gene therapy is one of promising approaches to cancer gene therapy. Tissue or tumor specific promoters and replication competent viral vectors have gained much interest in recent years. In this study we report the construction of a new replication competent bicistronic adenoviral vector carrying cytosine deaminase (CD) gene driven by L-plastin promoter (AdLpCDIRESE1a) and invitro cytotoxicity and in vivo efficacy in an animal model of colon cancer. A similar vector driven by CMV promoter has also been constructed (AdCMVCDIRESE1a). While AdLpCDIRESE1a showed significant cytotoxicity in tumor cells of breast (MCF-7), colon (HTB-38) and ovary (ovcar 5), no significant toxicity was seen in human mammary epithelial cells (HMEC). Addition of 5-fluorocytosine (5FC) significantly increased the cytotoxicity of both AdLpCDIRESE1a and replication incompetent vector (AdLpCD). However, no significant cytotoxicity was observed with the additon of 5FC in mammary epithelial cells. The tumor cells infected with AdLpCDIRESE1a vector, also showed significant by stander effect even in the ratio of 0.25 of infected cells. The injection of AdLpCDIRESE1a to the subcutaneous tumor nodules along with peritoneally administered 5-FC, decreased the tumor size and growth rate. Also this group of mice lived much longer than the other groups including viral vector alone and AdLpCD \pm 5-FC. The impressive results of in-vitro cytotoxicity and encouraging results of in-vivo testing of the our newly constructed AdLpCDIRESE1a suggest the potential use of the vector for intracavitary therapy of peritoneal carcinomatosis.

INTRODUCTION

Although only a small number of cancer gene therapy trials have been completed, and few still successful, gene therapy is regarded as one of the most exciting new directions in cancer treatment. Many of these trials have used adenoviral vectors to carry prodrug activation transcription units to sensitize the cancer cells to chemotherapy agents without generating unnecessary side effects on the normal tissues of the body. Among the prodrug strategies, adenoviral vectors carrying the *Escherichia Coli* or yeast cytosine deaminase (CD) gene (1), which catalyzes the conversion of the relatively harmless drug, 5-Fluorocytosine (5FC) into the cytotoxic agent, 5-Fluorouracil (5FU), is one of the most frequently used because of the following advantages: when generated in sufficient quantities, 5FU can kill both dividing as well as non dividing cells, due to its incorporation into RNA as well as DNA (2,3).

Adenoviral vectors are among the most widely used vector systems for the introduction of genetic elements into somatic cells for cancer gene therapy. The limitations of the adenoviral vectors include the stimulation of a vigorous immune response, although in cancer gene therapy, this may not always be a disadvantage. Adenoviral vectors have some advantages, which other vector systems do not share: transgene expression in non-dividing cells, and a broad range of cells which it infects. The ability to infect host cells, normal and cancer cells, and to express the therapeutic transgene in dividing as well as non-dividing cells, is a major cause of toxicity (4,5). The use of tumor or tissue specific transcription promoters is one of the current approaches to solving this problem (6,7). Accordingly, our laboratory has constructed adenoviral vectors using the L-plastin promoter, which is a tumor specific promoter, which drives the expression of therapeutic transgenes in tumor cells but not in normal cells (8). Experiments carried out by Xue yuan Peng of our laboratory has recently shown a tumor specific suppressive effect of the L-

plastin driven CD transcription unit carried by replication deficient adenoviral vectors to ovarian, breast and bladder cancers (9).

In the experiments summarized in this report, our goal was to study the use of replication competent and incompetent L-plastin-driven adenoviral CD vectors in normal and tumor cell lines, and therapeutic efficacy of the 5-FC/replication competent and incompetent L-plastin-driven CD vector gene therapy in an animal model of colon cancer.

MATERIALS AND METHODS

Cells

The human breast cancer cell line (MCF-7), the transformed human kidney cell line (HEK293) and the human colon cancer cell line (HTB-38) were purchased from American Type Culture Collection (ATCC). The human epithelial ovarian cancer cell line (Ovcar-5) was obtained from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. The human mammary epithelial cell line (HMEC) was purchased from Bio-Whittaker, Walkersville, MD. The MCF-7, Ovcar-5, HEK293 cell lines were grown in Dulbecco's Eagle Medium including 10% heat-inactivated newborn calf serum (NBC) and HTB-38 cells were grown in McCoy's 5A modified medium supplemented with 10% NBC. The HMEC cell line was grown in a growth medium supplemented with hydrocortisone (0.5ug/ml), insulin (5 ug/ml), epidermal growth factor (10 ng/ml) and bovine pituitary extract (26 ug/ml). All the cell lines were maintained in a 5% CO₂, humidified tissue culture incubator at 37°C. The HTB-38 cell line was tested for mouse hepatitis virus before inoculating the mice.

Construction of adenoviral vectors

Wild type Adenovirus type 5 (Ad5WT) was obtained from ATCC. A replication-deficient adenoviral vector carrying the L-plastin driven CD gene (AdLpCD) was engineered in our laboratory in a previous work (8,9). In the work described in this paper, a new replication competent bicistronic adenoviral vector carrying the L-plastin driven CD and E1a genes in a single continuous bi-cistronic transcription unit was constructed. The CD gene was synthesized by PCR from the pShuttleLp-CD plasmid using the primers XhoI (ccgctcgagaggctaagtgcgaat) and XbaI (gctctagattaccgtttgtaatcgat). The IRES (intra ribosomal entry site) sequence, obtained by PCR from pCITE (2+) with the primers SpeI (ggactagtggttatttccaccatattgccgt) and SalI (acgcgtcgacggtattatcatcgtgttttca) was inserted between the XbaI/SpeI and SalI restriction sites. An 2.4-kb fragment of the L-plastin promoter, truncated by Injae Chung of our laboratory from the 5-kb promoter extending from the nucleotide -2265 of the 5' region of the L-plastin promoter to +18 bp from the transcription initiation site of the L-plastin gene (8), was inserted between NotI and XhoI of pShuttle. This vector is designated AdLpCDIRESE1a. After linearizing the constructed plasmid with PmeI, it was cotransformed into the E.coli strain BJ5183 with pAdEasy-1 viral DNA plasmid for homologous translocation. Another bicistronic virus with the CMV tumor non-specific promoter, instead of the L-plastin promoter, was constructed. This vector is designated AdCMVCDIRESE1a. The maps of the newly constructed replication competent viruses are shown in Figure 1. Following the validation of the structure of the recombinant AdLpCDIRESE1a and ACMVCDIRESE1a by restriction digest, the vectors were transfected into HEK293 cells where the E1 gene is complemented in vivo (10).

The number of infectious adenoviral particles in the stocks of both AdLpCD and AdLpCDIRESE1a, expressed as plaque-forming units (pfu), was determined by limiting dilution assay of plaque formation in HEK293 cells.

Analysis of the expression of the CD gene in the AdLpCDIRESE1a Vector

The expression of the CD gene in the bicistronic transcription units of the AdLpCDIRESE1a vector was measured by extracting RNA from cells infected with the vector and then use of the RT-PCR of this mRNA to generate cDNA for molecular weight analysis. The primers for this reaction were: XhoI (ccgctcgagaggctaattgcgaat) and XbaI (gctctagattaccgtttgtaatcgat), which come from the CD gene. The predicted molecular weight of the CD fragment generated by this PCR reaction is around 1.2 Kb.

Cytopathic effect of the vectors

The HTB-38, MCF-7, Ovar-5 and HMEC cells were seeded at a density of 200,000 cells/well in 6-well plates. 24 hrs later, the cells were infected by exposure to the following vectors: Ad5WT, AdLpCD and AdLpCDIRESE1a and AdCMVCDIRESE1a at various MOI. Following the culture of the infected cells for an additional 4 days, the cells were examined for cytopathic effect by light microscopy.

Functional analysis of the CD gene in the Adenoviral Vector Backbone:

The HTB-38, MCF-7, Ovar-5 and HMEC cell lines were seeded at a density of 200,000 cells/well in 6 well plates. The cells were then exposed to the AdLpCD and AdLpCDIRESE1a at the following MOI (0, 0.1, 1, 10, 30, 80). 24 hrs later 500uM 5-fluorocytosine (5-FC, Sigma Chemical Co) was added to the cells. Following the incubation of the cells for an additional 4 days, the cells were trypsinized and the surviving cells counted with trypan blue exclusion test. The percentage of surviving infected cells was calculated by taking the ratio of the surviving cells incubated at 0 MOI as 100%.

Bystander effect assay

Following exposure of the cell line to the vector under conditions which would lead to infection of 100% of the cells (MOI of 80 with AdLpCD and AdLpCDIRESE1a viruses), the infected cell lines HTB-38, MCF-7, Ovar-5 and HMEC were mixed individually with the corresponding uninfected cells at the following ratios of infected to uninfected cells: 0, 0.05, 0.25, 0.50, and 1.0. The seeding density of the total number of cells was 2×10^6 cells/well into six-well plates. 24 hrs later, 500 μ M 5-FC was added. Following the incubation of the cells for an additional 4 days, the cells were trypsinized and the surviving cells counted with the trypan blue exclusion test.

Animal studies

The HTB-38 cells (3×10^6) suspended in PBS were injected s.c. in 35 nude/nude mice, purchased from Charles River Labs, Wilmington. Following sufficient time to allow for the development of the tumor nodule to a size in the 50 cumm range, the mice were randomly divided into 5 groups and 10^8 pfu of the AdLpCDIRESE1a and AdLpCD vectors were injected intratumorally (i.t.) for two groups of 7 mice for each of the treatment groups, and with PBS for the control group. Then, 500 mg/kg of 5FC was injected intraperitoneally into experimental groups of animals in which the sensitization of the infected tumor cells to 5FC by CD was to be studied, and with equal amounts of PBS into animals in which the growth of the tumor nodule in the absence of treatment was to be studied, once a day, for 7 days. Then, tumor sizes were measured every 6 days. Tumor volumes were calculated by the formula 'volume = length x (width²/2)' (9).

Statistical analysis

Results of the invitro cytotoxicity tests were evaluated by a non-linear regression method and the maximum cytotoxicity of the viruses were compared by the Student's t test. One-way ANOVA (with LSD post-hoc comparisons) and Mann-Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analysis was performed according to Kaplan-Meier method and the log-rank test was used for survival comparisons.

RESULTS

Expression of the CD gene in vector exposed tumor cells.

The MCF-7, Ovar-5 and HTB-38 cell lines were seeded at a density of 200,000 cells/well in 6-well plates. 24 hrs later, these cells were infected by the Ad5WT, AdLpCD and AdLpCDIRESE1a and AdCMVCDIRESE1a vectors. Following an infection period of 12 and 16 hours, the cells were collected by trypsinization and washed with PBS. Total RNA was then isolated and cDNA was then generated using the primers specific for the CD coding transcripts. CD gene was synthesized and amplified from the mRNA of the gene by RT-PCR. The bands for CD gene, obtained by 1% agarose gel electrophoresis, are shown in Figure 2. The bands produced in Figure 2 show that the expected molecular weight was obtained from the amplification of the RNA from the CD vector cells, whereas it was not seen in the RNA from the cells infected by the control vector.

Analysis of cytopathic effects generated in normal mammary epithelial cells in cell lines exposed to newly constructed viruses.

The cytopathic effects (CPE) generated by exposure to the Ad5WT, AdCMVCDIRESE1a, AdLpCDIRESE1a, and AdLpCD vectors were studied in the following tumor cell lines: MCF-7, Ovar-5 and HTB-38, and in explants of normal mammary epithelial cells (HMEC). Replication competent viruses (AdWT, AdLpCMVCDIRESE1a and AdLpCDIRESE1a) produced a strong CPE following 4 days of infection in tumor cells (data not shown). The wild type adenovirus (AdWT) and the CMV driven viruses (AdCMVCDIRESE1a) produced a strong CPE on HMEC, whereas the Lp-driven viruses (both replication competent and non-competent) had no or minimal CPE (Figure 3). These studies showed that the L-plastin promoter was tumor specific in its generation of a cytopathic effect in mammalian cells.

Analysis of the in vitro cytotoxicity test generated by vectors at different MOI in tumor cell lines.

In this study, invitro cytotoxicity tests were carried out with both replication competent and incompetent L-plastin-driven vectors. As shown in Figure 4, the cells exposed to the AdLpCDIRESE1a vector at showed significant cytotoxic effect starting at the dose of 1 MOI in tumor cells (Figure 4). The maximum predicted cytotoxic effect of the replication competent AdLpCDIRESE1a vector (with 5FC treatment) was seen at 80 MOI. The percentage of cells killed seen in the MCF-7 and HTB-38 cell lines at 80 MOI (70.9% and 66.1%) was greater than that seen in the Ovar-5 cell line (46.5%). However, the maximum cytotoxic effect of the AdLpCD replication incompetent vector at 80 MOI with 5FC treatment was 19.3% in MCF-7, 21.6% in HTB-38 and 16.1% in Ovar-5 cells (see Fig. 4). The cytotoxic effect of replication-competent virus was significantly higher than that of the non-competent virus in all studied tumor

cell lines ($p < 0.0001$). In contrast to the experience with the tumor cell lines, the rate of the surviving HMEC cells exposed to vectors at 80 MOI was more than 80 % after 5 days of the infection for both replication competent and non-competent vectors when they were driven by the L-plastin promoter.

The maximum predicted cytotoxicity after addition of 5-FC to the AdLpCDIRESE1a vector was 91.3% in MCF-7, 94.9% in HTB-38 and 87.3% in Ovar-5 cells. Addition of 5-FC significantly increased the cytotoxicity in all tumor cells ($p < 0.001$, $p < 0.01$ and $p < 0.0001$, respectively). 5-FC, also caused significantly more cell death in MCF-7, HTB-38 and Ovar-5 tumor cell lines infected with non-competent vector when compared to vector alone (58.6% $p < 0.0001$, 57.0% $p < 0.01$ and 67.7% $p < 0.001$, respectively). The change in maximum predicted cytotoxicity of the vector in HMEC cell line after addition of 5-FC was not significant for both types of vectors (for replication competent virus; 13.8% vs. 19.2 %, $p > 0.05$ and for non-competent virus; 7.4% vs. 11.1%, $p > 0.05$). This data also supports the tumor selective expression of CD gene carried by newly constructed vectors.

Analysis of the cytotoxic effect of infected cells on uninfected cells

To evaluate the effect of 5-FU generated in and released from the CD vector infected cells on the uninfected cells by the CD catalyzed conversion of 5FC into 5FU and the release of 5FU from the infected cells, we generated mixtures of the cells infected with the AdLpCDIRESE1a and AdLpCD vectors under conditions which lead to infection of 100% of the cells, with uninfected cells at the following ratios of infected to the uninfected + infected cells: 0, 0.05, 0.1, 0.25, 0.50, and 1.0. The mixtures were plated in 6 well plates and then exposed to 5-FC at 500uM. The effect of the 5-FU released from the infected cells on the vector non infected cells was seen starting at a ratio of infected to non-infected + infected cells of 0.25 (see Figure 5).

More than half of the cells were dead even when the ratio of infected cells was less than 25%. The significant by-stander effect augments the cytolytic effect of the CD vectors is greater in the presence of 5FC than in its absence, and the vector with the Lp-CDIRESE1a bicistronic transcription unit was more toxic than the Lp-CD vector. Thus, the addition of the E1a transcription unit to that of the CD gene appeared to increase the cytotoxic effect of the vectors on tumor cell lines. Finally, all of the effects of the vectors appeared to be tumor specific when the L-plastin promoter was used to drive the therapeutic transcription units, on the basis of the comparison of the effect of the L-plastin and CMV driven vectors on normal mammary epithelial cells as compared to breast cancer cell lines.

The efficacy of the AdLpCDIRESE1a replication competent virus in in-vivo model

The efficacy of replication-competent and non-competent vector/5-FC systems was tested in nude mice bearing tumor nodules of subcutaneously injected the human colonic cancer HTB-38 human colonic cancer cell line. On the eighth day of the injection of HTB-38 cells, the tumor volumes were measured and the mice were randomly divided into 5 groups: the mice injected intratumorally with the AdLpCDIRESE1a vector and then treated intraperitoneally with 5-FC, the mice injected intratumorally with the AdLpCDIRESE1a vector without 5FC treatment, the mice injected intratumorally with the AdLpCD vector followed by intraperitoneal 5-FC treatment, the mice injected intratumorally with the AdLpCD vector without 5FC treatment, and control mice injected intratumorally with PBS. The average volume of tumor nodules in the control and AdLpCDIRESE1a treated groups on the first day of treatment (day 0) were lower than in the other groups (see Figure 6). The mice in all groups were followed until the animal was sacrificed due to large tumor size or until the end of the 44 days of observation period. Most of

the mice, which were alive at the end of the observation period, were sacrificed at the end of the observation period (44th day of virus injection) because of the large size or the tumor nodules.

There were 4 partial remissions (4/7) in the AdLpCDIRESE1a+5FC treated group at the end of the first week of the treatment. The average tumor volumes of this group significantly decreased during the first week ($p=0.046$). However, the tumor nodules of the responding mice started to grow after 2 weeks of drug administration. Nevertheless, the tumor growth rate in this group was significantly lower than that of the other groups during whole study period ($p<0.05$). Tumor volumes of the AdLpCDIRESE1a+5FC treated group were significantly lower than that of the AdLpCDIRESE1a, AdLpCD+5-FC and control groups during the observation period ($p<0.05$) (Figure 6). There were no objective tumor responses in other groups. However, tumor growth rate of AdLpCDIRESE1a group was significantly lower than control and AdLpCD groups ($p<0.01$). There was no significant tumor response in the AdLpCD+5-FC group. Tumor volumes of this group also did not significantly differ than AdLpCD and control groups (Figure 6).

The mice in the AdLpCDIRESE1a+5-FC group lived significantly longer than the other groups ($p<0.02$) as shown in Figure 7. While the median survival in this group was not reached during the observation period, it was 41, 36, 36 and 22 days for AdLpCDIRESE1a, AdLpCD+5-FC, AdLpCD and control groups. All of the virus-injected groups lived longer than the mice in the control group ($p<0.01$).

DISCUSSION

In this report, we present the analysis of a series of adenoviral vectors designed to explore the potential of using prodrug activation transcription units driven by a tumor specific

promoter (L-plastin). The cytotoxic effect of the vector carrying both the E1A and the CD transgenes was greater than the vector which contains only the CD gene prodrug activation transcription unit without the E1a gene, both in in vitro cell line experiments as well as in experiments in human tumor xenograft models.

Pro-drug activation transcription unit gene therapy is one of many new and promising approaches to cancer treatment. Previous reports have studied the tumor suppressive effect of adenoviral vectors carrying the CD gene/5-FC system on various tumor cell lines and in vivo models (2,11). However, most of these reports are focused on replication incompetent viral systems. The infectivity of normal as well as tumor cells by the adenoviral vector has represented a disadvantage for adenoviral vectors since the expression of the therapeutic transgenes in the normal cells generates side effects. In order to overcome this limitation, many groups have been focusing on tumor or tissue specific gene promoters to reduce side effects. Our laboratory has recently reported experiments involving vectors carrying the tumor specific L-plastin driven promoter (8,9).

The use of replication competent viral vectors is a relatively new approach in gene therapy of malignant diseases. The goal of this strategy is the direct killing of the target tumor cell by oncolytic effect of the virus (12). Therefore, the insertion of prodrug activation transcription units driven by the L-plastin promoter into conditionally replication-competent vectors was predicted to increase the therapeutic effect without increasing toxicity.

In this study, we constructed a conditionally replicative bicistronic adenoviral vector carrying both Lp-driven CD and E1a genes, and placed these transcription units under the control of the tumor specific L-plastin promoter. Our newly constructed vector showed significant a CPE on breast (MCF-7), colon (HTB-38) and ovary (Ovcar-5) carcinoma cell lines (data not shown), which was greater than that of a replication incompetent vector. Importantly, both replication

competent and incompetent vectors, which carried CD/E1a or CD transcription units under the control of the L-plastin tumor specific promoter caused no significant CPE on human mammary epithelial cells (HMEC). However, wild type adenovirus and similarly constructed replication competent CMV driven adenoviral vectors caused significant CPE to the HMEC by the 4th day after in initial exposure to the vector (Figure 3). These data show that the L-plastin driven CD or CD/E1a vectors are selectively toxic to the tumor cell lines without being toxic to the normal mammary cell explants.

MCF-7 and HTB-38 cells were found to be more sensitive to the new vector than Ovar-5 cells ($p < 0.01$ and $p < 0.02$, respectively). The cytotoxicity effect of replication incompetent vectors was minimal in both tumor cell lines and in normal cells. The addition of 5-FC to both vectors significantly increased the cytotoxicity in tumor cells (Figure 4). The conditionally replication competent vectors+5-FC killed almost all MCF-7 and HTB-38 cells. In both replication competent and replication incompetent virus/5-FC systems, active drug released from the infected cells may cause significant cell death among the uninfected cells. Our data show that more than half of the cells were dead even the ratio of infected cells was less than 25%. This significant by-stander effect seems to increase the efficacy of this virus/prodrug therapeutic system.

The significant cytotoxicity seen with the in vitro assays with tissue culture tumor cell lines led to the testing of the AdLpCDIRESE1a/5FC system in an in vivo human tumor xenograft model. We therefore established an in vivo colon carcinoma model of nude/nude mice with subcutaneously injected HTB-38 cells. In our model, the AdLpCDIRESE1a viral vector is injected intratumorally into subcutaneous nodules of the human colonic cancer cell line, HTB-38 followed by intraperitoneal injections (twice a day) of 5FC in quantities sufficient to generate a peak concentration of 500 micromolar. The injection of the AdLpCDIRESE1a vector in the

absence of 5FC did not cause tumor response but decreased the tumor growth rate. Addition of 5FC yielded approximately 60% objective tumor response rate along with a decrease in tumor growth rate. The mice in this group treated with the AdLpCDIRESE1a/5FC system lived significantly longer than the mice in other groups. No tumor response was seen in the AdLpCD+5FC treated group of mice. This is thought to be due to the provision of insufficient viral particles to the tumor nodules. However, this problem can be overcome by repeated injections of viral vector and prolonged administration of 5FC.

The impressive results of the invitro cytotoxicity tests along with the longer survival of the conditionally replication competent vector/5FC injected mice suggests that the promising role of the bicistronic vectors are more effective than the replication incompetent CD vectors and that the L-plastin promoter can make the effect of these vectors tumor specific. This vector system appears to have potential for intracavitary therapy of peritoneal studding in ovarian cancer patients.

ACKNOWLEDGEMENTS

The authors acknowledge support from the Bush Lekemia Research Fund, the Breast Cancer Research Foundation, and the US Army Breast Cancer Research Program (DAMD 17-99-1-9457), and a laboratory supply fund from the Sidney Kimmel Cancer Center. Hakan Akbulut recognizes a fellowship from the National Cancer Institute and TUBITAK.

REFERENCES

1. Lal S, Lauer UM, Niethammer D, et al. Suicide genes: past, present and future perspectives. *Immunol. Today* 2000; 21:48-54.
2. Hirschowitz EA, Ohwada A, Pascal WR, et al. In vivo adenovirus-mediated gene transfer of the Escherichia coli cytosine deaminase to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum. Gene Ther.* 1995; 6:1055-1063.
3. Bentires-Alj M, Hellin A-C, Lechantewur C, et al. Cytosine deaminase suicide gene therapy for peritoneal carcinomatosis. *Cancer Gene Ther.* 2000; 7:20-26.
4. Shenk TE. Adenoviridae: the viruses and their replication. In: Knipe DM, Howley PM (eds), *Field's Virology*, Forth edition, Philadelphia, Lippincott Williams&Wilkins; 2001: 2265-2300.
5. Joss K, Turka LA, Wilson JM. Blunting of immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig. *Gene Ther.* 1998; 5:309-319.
6. Nettelbeck DM, Jerome V, Muller R. Gene therapy: designer promoters for tumor targeting. *Trends Genet.* 2000; 16:174-181.
7. Pang S, Taneja S, Dardashti K, et al. Prostat tissue specificity of the prostat-specific antigen promoter isolated from a patient with prostat cancer. *Hum. Gene Ther.* 1995; 6:1417-1426.
8. Chung I, Peter ES, Crystal RG, Pizzorno G, Deisseroth A. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Ther.* 1999; 6:99-106.

9. Peng XY, Won JH, Rutherford T, et al. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res.* 2001; 61:4405-4413.
10. He T-C, Zhou S, DaCosta LT, et al. A simplified system for generating recombinant viruses. *Proc. Natl. Acad. Sci. USA* 1998; 95:2509-2514.
11. Kievit E, Nyati MK, Ng E, et al. Yeast Cytosine Deaminase Improves Radiosensitization and Bystander Effect by 5-Fluorocytosine of Human Colorectal Cancer Xenografts. *Cancer Res.* 2000; 60:6649-6655.
12. Alemany R, Balague C, Curiel DT. Replicative adenoviruses for cancer therapy. *Nature Biotechnol.* 2000; 18:723-727.

FIGURE LEGENDS

Figure 1. The map of a newly constructed conditionally replication competent adenoviral vector.

A: AdLpCDIRESE1a; B: AdCMVCDIRESE1a

Figure 2. Expression of CD gene in MCF-7 cells. 1: λ DNA Hind III fragments, 2: control RNA,

3: AdLpCDIRES plasmid, 4: wild type adenovirus, 5: no virus infection, 6: AdLpCD,

7: AdLpCDIRESE1a (12 hour infection), 8: AdLpCDIRESE1a (16 hour infection), 9:

AdCMVCDIRESE1a, 10: 1kb DNA marker, a: with reverse transcriptase (RT), b: without RT.

Figure 3. Cytopathic effect seen in human mammary epithelial cell line (HMEC) infected with;

A. wild type adenovirus; B. AdCMVCDIRESE1a; C. AdLpCDIRESE1a; and D. AdLpCD.

Figure 4. Results of an in vitro cytotoxicity test. A: MCF-7 cells; B: HTB-38 cells; C: Ovar-5

cells. D. HMEC cells. — :AdLpCDIRESE1a+5-FC;

⧫ : AdLpCDIRESE1a; ⧫ — :AdLpCD+5-FC; ⧫ — :AdLpCD. (Markers represent observed and lines predicted data).

Figure 5: Results of by-stander effect assay. A: MCF-7 cells; B: HTB-38 cells; C: Ovar-5 cells;

D: HMEC cells. (⧫ — :AdLpCDIRESE1a+5-FC; ⧫ — :AdLpCD+5-FC. (Markers represent observed and lines predicted data).

Figure 6. Effect of intratumoral injection of tumor nodules with adenoviral vectors on tumor growth.

Figure 7. Kaplan-Meier survival curves of nude mice with tumor nodules from HTB-38 colon cancer.